Biochemical characterization of cytochrome P450 enzyme (CYP6P9) involvement in insecticide resistance in the major malaria vector *Anopheles funestus*

Tonderayi Sylvester Matambo

'A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy'

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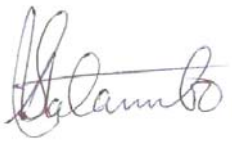
DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.



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Abstract

Anopheles funestus is one of three major malaria vectors in Africa. During 2000 malaria cases increased in Kwazulu/Natal, South Africa. This was a result of pyrethroid resistance in this vector. Biochemical studies showed elevated levels of Cytochrome P450 (monooxygenases) in the resistant mosquitoes. Monooxygenases are one of the three major metabolic based resistance mechanisms used by mosquitoes as a defence mechanism against insecticides. Further studies identified one gene in the CYP6 class, CYP6P9, highly overexpressed. Microsomal cytochrome P450s were isolated from pyrethroid resistant (FUMOZ-R) larval midguts and headless adults. High enzyme activity of 7-ethoxycoumaorin was noted in both larval midguts and adults (FUMOZ-R) compared to susceptible (FANG) strain (3.6 and 6.4 fold increases respectively). HPLC analysis further confirmed that microsomal cytochrome P450s from FUMOZ-R were involved in permethrin (pyrethroid) detoxification. In order the understand the mode of action of CYP6P9 as a metabolizing enzyme, this study aimed to isolate and characterize full length CYP6P9 as well as develop a suitable expression system in *E. coli.* Sequence comparisons of full-length cDNA's between CYP6P9 from resistant and susceptible strains revealed several mutations. Redox partners Cytochrome b_5 and Cytochrome P450 reductase genes were also isolated, sequenced and cloned as these proteins plays an integral part in detoxification. These were almost identical between the two strains analysed.

A second cytochrome P450, CYP6P13, was isolated only from FANG genomic DNA. This gene shared a 93.7% identity with that of CYP6P9. Its absence in FUMOZ-R and FANG cDNA indicated that it was not expressed at the protein level. This gene is most likely the result of gene duplication. In order to enzymatically characterize the metabolic activity of CYP6P9, recombinant CYP6P9 protein was expressed in *E. coil*. Expression of CYP6P9 was problematic in *E. coli* even when the expression vector was changed from pIX4.0 to pCWori^{+} in the presence of a bacterial signal sequence (*ompA*). Expression was successful when eight of the first amino acid sequences were replaced by the 17-α hydroxylase sequence. This work is the first to provide evidence of permethrin detoxification by microsomal P450 from a resistant mosquito strain. It is also the first to express *An. funestus* P450 in *E. coli.*

Dedication

To my loving wife Denga Mokgadi Matambo and my parents Alexander Caps Matambo and Adeline Matambo. Thank you for all your support.

Publications and presentations based on the study

Publications:

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Matambo, T. S., Koekemoer, L. L. and Coetzee, M. Characterization of Cytochrome P450 (CYP6P9) from insecticide resistant malaria vector *Anopheles funestus. Poster presentation, National Institute for Communicable Diseases academic day, 27 -28 November 2006*.

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LIST OF SYMBOLS

α	:	alpha
γ	:	gamma
β	:	beta
ºC	:	degree celcius
%	:	percent or g/100 ml
Å	:	Ångström
µg	:	micrograms
µL	:	microlitre
µM	:	micromolar
Bp	:	base pair
Da	:	Daltons
g	:	gram
kDa	:	kilodaltons
L	:	litre
M	:	molar
mg	:	milligram
min	:	minutes
ml	:	millilitre
mM	:	millimolar
mol	:	mole
MW	:	molecular weight
ng	:	nanogram
nm	:	nanometer
nmol	:	nanomole
U	:	units
V	:	volts
v/v	:	volume per volume
w/v	:	weight per volume
x g	:	relative centrifugal force to gravity
RCF	:	relative centrifugal force
rpm	:	revolutions per minute

NOMENCLATURE

$(His)_6$	:	6x histidine – tag
$(NH_4)_2S_2O_8$	:	ammonium persulphate
AChE	:	acetylcholinesterase
ALA	:	δ-Aminolevulinic acid
AMP	:	ampicilin
BLAST	:	Basic Local Alignment Search Tool
BSA	:	Bovine serum albumin
CAM	:	chloramphenicol
cDNA	:	complementary DNA
CO	:	carbon monoxide
CHAPS	:	3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate
CPR	:	cytochrome P450 reductase
CYP	:	cytochrome P450 protein
Cytb_5	:	cytochrome b_5
DDA	:	dichlorodiphenyl acetic acid
DDD	:	dicofol, dichlorodiphenyl-dichloroethane
DDT	:	1,1,1-trichloro-2,2-bis-(4'-chlorophenyl)ethane
dNTP	:	deoxyribonucleoside triphosphate
DTT	:	dithiothreitol
EDTA	:	ethylenediamine tetra-acetic acid
FAD	:	flavin adenine dinucleotide
FMN	:	flavin mononucleotide
FANG	:	pyrethroid susceptible *An. funestus* from southern Angola
FUMOZ-R	:	pyrethroid resistant *An. funestus* from Mozambique
gDNA	:	genomic DNA
G6P dehydrogenase	:	glucose -6-phosphate dehydrogenase
GABA	:	gamma (γ) aminobutyric acid receptor
GS-SG	:	glutathione oxidized
GST	:	glutathione-S-transferases
HPLC	:	High Performance Liquid Chromatography
IPTG	:	isopropy-1-thio-beta-D-galactopyranoside
kdr	:	knockdown resistance
KCl	:	potassium chloride
KH_2PO_4	:	potassium phosphate, monobasic
K_2HPO_4	:	potassium phosphate, dibasic
LB	:	Luria Bertani
Mean ± S.E	:	Mean ± Standard Error
MgCl	:	magnesium chloride
Na_2HPO_4	:	disodium hydrogen phosphate
NADPH	:	nicotinamide adenine dinucleotide phosphate (reduced)

NaCl	:	sodium chloride
Ni-NTA	:	nickel-nitrilotriacetic acid
OD	:	optical density
P450	:	Cytochrome P450
PBO	:	piperonyl butoxide
PBS	:	phosphate buffer saline
PCR	:	polymerase chain reaction
PMSF	:	phenylmethylsulfonyl fluoride
SDS	:	sodium dodecylsulphate
SDS-PAGE	:	SDS-polyacrylamide gel electrophoresis
SRS	:	substrate recognition site
TAE	:	Tris-acetate EDTA electrophoresis buffer
TB	:	Terrific Broth
TBS	:	Tris buffer saline
TBST	:	TBS-Tween
TSE	:	Tris-acetate sucrose EDTA buffer
Tris	:	Tris (hydroxymethyl) aminomethane
X-gal	:	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

LIST OF THE 20 AMINO ACIDS AND NUCLEIC ACIDS

Listed below are the International Union of Pure and Applied Chemistry (IUPAC)
and
International Union of Biochemistry and Molecular Biology (IUBMB) nomenclature
Of the 20 amino acids and nucleic acids.

AMINO ACDS

Name	Abbreviation	code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

NUCLEIC ACIDS

Name	Code
Adenosine	A
Thymidine	T
Guanosine	G
Cytidine	C

Chapter one

Literature review

1 General Introduction

1.1 Importance of Malaria

Malaria is one of the oldest diseases known to man and was thought to be caused by the bad smelling gases from marshy places hence the name *mal' aria,* "bad air" (Garnham, 1966). In 1880, Charles Louis Alphonse Laveran first illustrated that malaria was caused by a parasite. This was proven by the presence of parasites in the blood of malaria patients (Garnham, 1966; Harrison, 1978). Human malaria is caused by four major *Plasmodium* species: *Plasmodium vivax, P. malariae, P. ovale,* and *P. falciparum*. *Plasmodium falciparum* is the most severe of all (Garnham, 1966). The parasite is transmitted to the human host by an infected blood feeding *Anopheles* female mosquito. The transmission of malaria by mosquitoes to man was demonstrated by an Italian group, Amico Bignami, Giovanni Battista Grassi and Guiseppi Bastianelli in 1898. They achieved this by allowing infected mosquitoes to transmit *P. falciparum* to a volunteer (Garnham, 1966). Sir Ronald Ross at the same time confirmed this while working on bird malaria which earned him a Nobel Prize in 1902 (Harrison, 1978).

Mosquitoes belong to the order *Diptera* (they have only one pair of functional wings) and undergo a holometabolous life cycle (a three development stage which includes: larvae, pupae, adult (imago) (Service, 1980).

In Africa, malaria has a major health impact especially south of the Sahara where it influences social and economic growth (WHO, 2001). The estimated cost to Africa alone is over US$ 12 billion per year (WHO, 2001). At least 300 million global cases of malaria are reported with more than a million deaths annually (WHO, 2001). Africa south of the Sahara is the most affected region as a result of the high prevalence of *Plasmodium falciparum* as well as the presence of three very efficient malaria vector species (WHO, 2001).

1.2 Malaria vectors

Mosquitoes have 34 genera that belong to three subfamilies, Anophelinae (anophelines), Toxorhynchitinae and Culicinae (culicines). Mosquitoes belonging to the genera *Anopheles, Culex, Aedes, Mansonia, Haemagogus, Sabethes and Psorophora* are important due to the fact that they acquire blood meals (Service, 1980). The bulk of malaria transmission in Africa is caused by three major vectors, *An. gambiae s.s, An. arabiensis* and *An. funestus s.s* (Gillies and De Meillon, 1968; White, 1974; Gillies and Coetzee, 1987). *Anopheles gambiae s.s* and *An. arabiensis* are members of the *An. gambiae* complex while *An. funestus s.s* is a member of the *An. funestus* group.

1.2.1 *Anopheles funestus* group

The first classification of *An. funestus* group was based on morphology. Species were distinguished at various life stages: eggs, larvae and adults. Based on morphological similarities nine species were grouped into the *An. funestus* group. These include: *An. funestus* Giles, *An. vaneedeni* Gillies and Coetzee, *An. parensis* Gillies, *An. rivulorum* Lesson, *An. leesoni* Evans, *An. aruni* Sobti, *An. brucei* Service, *An. confusus* Evans and Leeson and *An. fuscivenosus* Leeson (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). A new species was identified in Cameroon that was very similar to *An. rivulorum,* hence it was named *An. rivulorum-like sp* (Cohuet *et al.,* 2003). Species within the group are not easily distinguished morphologically. Koekemoer *et al* (2002) developed a molecular species-specific polymerase chain reaction (PCR) assay that identifies five members of the *An. funestus* group in Africa, which are *An. funestus*, *An. vaneedeni*, *An. rivulorum*, *An. leesoni* and *An. parensis*.

Re-classification of genus *Anopheles* was suggested by Harbach (2004). This classification arose as a result of molecular studies. It involves removal and addition of species as well as changes within the hierarchical organization and supraspecific group composition. Classification based on molecular techniques involved sequence comparison within species on i) ITS2 (Nuclear ribosomal loci, internal transcribed spacer 2), ii) D3 (third domain of the 28S subunit of ribosomal DNA) and iii) COII (cytochrome oxidase subunit II) (Garros, *et al.,* 2005a; Garros *et al.,* 2005b; Chen *et al.,* 2006; Singh, *et al.,* 2006). The *An. funestus* group was subgrouped, *An. confuses* was added to the *An. funestus* subgroup along with *An. aruni, An. funestus, An.*

parensis and *An. vaneedeni*. *Anopheles leesoni* was added to the Minimus subgroup while *An. rivulorum, An. rivulorum-like sp, An. brucei* and *An. fuscivenosus* were grouped in the *An. rivulorum* subgroup (Garros, *et al.,* 2005a; Garros *et al.,* 2005b).

Species in the group are small, dark mosquitoes that are known to breed in permanent water bodies such as swamps and river systems and are therefore less affected by rainfall seasons. They have low tolerance to saline water and are never found in brackish water (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). *Anopheles funestus* is a major transmitter of *Plasmodium falciparum* and in some cases more so than *An. gambiae* (Temu *et al*., 1998; Cohuet *et al*., 2004). In studies carried out by Cohuet *et al* (2004) it was shown that *An. funestus* was a major transmitter of *P. falciparum* in a village of savannah-forest, Cameroon. It accounted for a total malaria transmission rate of about 88%. *Anopheles funestus* was also identified as the major malaria vector in an irrigated agricultural area in a sugarcane and savannah village of Tanzania (Ijumba *et al*., 2002).

Anopheles funestus is highly anthropophilic (biting man) and endophilic (resting indoors). This behaviour renders its susceptible to indoor spraying of residual insecticides (Gillies and De Meillon, 1968). Species within the group tend to compete for habitats and *An. funestus* is usually the dominant species. When *An. funestus* was eliminated from the Pare area in Tanzania by house spraying, *An. rivulorum* population increased. The same was noted in Kenya coast when *An. parensis* occupied villages that were sprayed to eliminate *An. funestus*. *Anopheles*

funestus are slow in re-invading areas they were once eliminated from by house spraying (Gillies and De Meillon, 1968).

1.3 Malaria in Southern Africa

In southern Africa the countries greatly affected by malaria epidemics are Angola, Botswana, Malawi, Mozambique, Namibia, South Africa, Swaziland, Zambia and Zimbabwe (WHO-SAMC, www.malaria.org.zw). All three major vectors (*An. arabiensis, An. gambiae s.s* and *An. funestus s.s.*) are found in this region. *Plasmodium falciparum* has been reported as the prevailing malaria parasite with an annual death toll of about 200,000 in southern Africa (WHO-SAMC, www.malaria.org.zw). The malaria transmission areas are categorized into three classes, which are:

1. Malaria free – these are areas where malaria transmission does not occur.
2. Unstable (epidemic-prone) transmission – these are areas in which malaria occurs seasonally and there is a high risk of morbidity and mortality if measures are not taken to control the outbreak of malaria.
3. Stable (endemic) transmission – these are areas in which malaria is throughout the year and there is a very high morbidity and mortality rate (WHO-SAMC, 2002).

South Africa mainly has malaria free zones and a small portion on the border region shows unstable seasonal malaria. DDT (Dichloro-Diphenyl-Trichloroethane) was introduced for indoor residual spraying (IRS) to combat malaria during the 1940's.

The South Africa malaria control programme changed because: 1) the locals were refusing to have their houses sprayed with DDT because of the ugly marks it left on walls and it agitates bedbugs to bite more, 2) deltamethrin (pyrethroid) replaced DDT because it was as effective and long lived as DDT, 3) environmental concerns towards DDT (Attaran and Maharaj, 2000; Coetzee, 2005). The use of pyrethrum in the control of mosquitoes was proven effective as early as the 1930's (De Meillion, 1936). Various factors influence the success of malaria control such as drug resistance in the parasites and insecticide resistance in the vectors (WHO-SAMC, www.malaria.org.zw). The impact of insecticide resistance on malaria transmission was clearly demonstrated in South Africa (Hargreaves *et al*., 2000). In Kwazulu/Natal, malaria cases increased from 4,117 in 1995 to 41,786 in 2000 (Figure 1.1), even though house spraying for malaria control was an ongoing practice. Studies conducted in 1999 showed that pyrethroid resistant *An. funestus* was a major contribution factor towards this increase of malaria cases (Hargreaves *et al*., 2000).

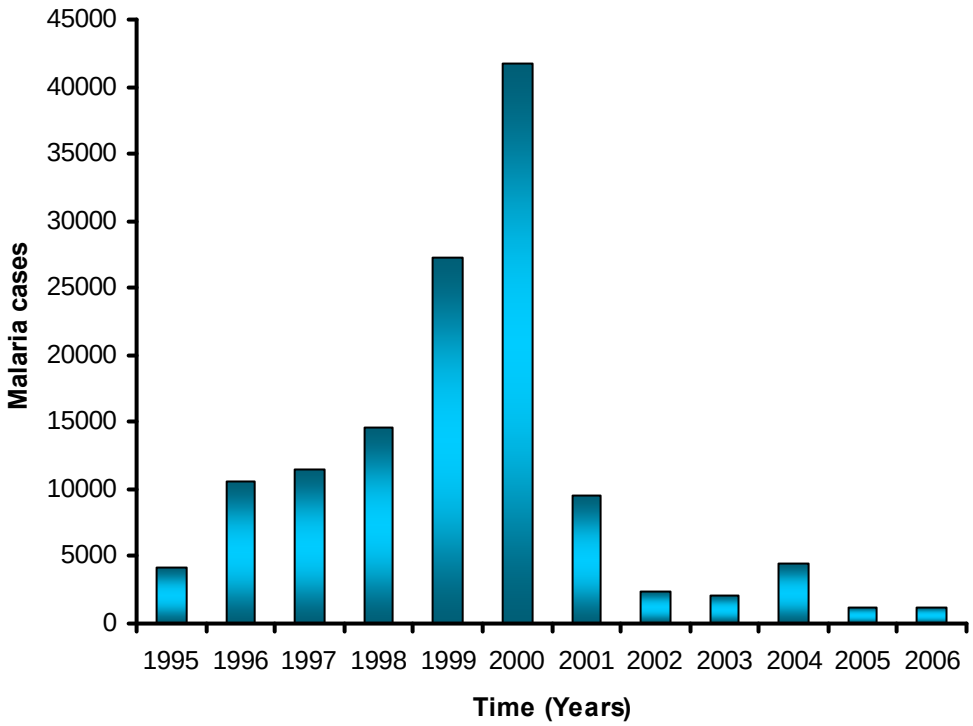


Figure 1.1: Malaria cases in Kwazulu/Natal 1995-2006, Department of Health, South Africa.

Pyrethroid resistance in *An. funestus* from South Africa was reported in the Ndumu area of northern Kwazulu/Natal bordering on southern Mozambique. A total of 5.4% of these mosquitoes tested were positive for *Plasmodium falciparum* circumsporozoite protein (Hargreaves *et al*., 2000). Resistance was confirmed by using three sets of pyrethroid bioassays, firstly the bottle bioassay that was developed by Brogdon and McAllister (1998). This assay uses a diagnostic dosage of permethrin (25µg/bottle) to detect resistant mosquitoes. The second assay used was the WHO test kit that uses 1% permethrin impregnated papers. The third bioassay involved residual house spraying using deltamethrin (0.05%) which was detected by window exit traps (WT) and pyrethrum knockdown spray catches (PSC). Brooke *et*

al (2001) used the WHO insecticide susceptibility test and found that *An. funestus* was resistant to pyrethroids (Deltamethrin, 0.05%; Lambdacyhalothrin, 0.05%) as well as the carbamate propoxur (0.1%). Biochemical assays concluded that mixed function oxidases (MFO) were the main mechanism of resistance (Brooke *et al.,* 2001). A drop to 1211 in 2006 was the result of the re-introduction of DDT for house spraying in 2000. The use of pyrethroids as an indoor residual spray (IRS) was retained for western-style homesteads while DDT was reintroduced for IRS in traditional homesteads (Figure 1.1) (Hargreaves *et al*., 2003).

1.4 Insecticide resistance

The WHO's official definition of resistance is the "development of an ability in a strain of organism to tolerate doses of toxicant which would prove lethal to the majority of individuals in a normal (susceptible) population of species" (cited by Zlotkin, 1999). Brown (1986) defines resistance mechanisms as follows: "Resistance is usually due to a detoxification of the insecticide due to mutant enzymes (isozymes) engendered by the resistance gene alleles, but some resistance may also be conferred by a reduced uptake of the toxicant". Resistance is brought about by various factors such as the extensive use of agricultural insecticides in malaria areas causing vectors to become more resistant to insecticides. Mosquitoes have developed two main mechanisms of resistance:

1. Target-site insensitivity which results from mutation(s) within the various insecticide target sites such as the sodium ion channel (also called knockdown

resistance (*kdr*)), gamma (γ) aminobutyric acid (GABA) receptor and acetylcholinesterase (AChE) gene (Mutero *et al*., 1994; Zhang *et al*., 1994; Martinez-Torres *et al*., 1998; Ranson *et al*., 2000; Hemingway *et al*., 2004; Weill *et al*., 2004).

2. Metabolic based resistance which involves elevated levels of detoxifying enzymes such as esterases, glutathione-S-transferases (GST) and monooxygenases (Hemingway and Ranson, 2000; Hemingway *et al*., 2004).

Below are the classes of insecticides used in malaria control and the target sites and enzyme associated in resistance (Figure 1.2).

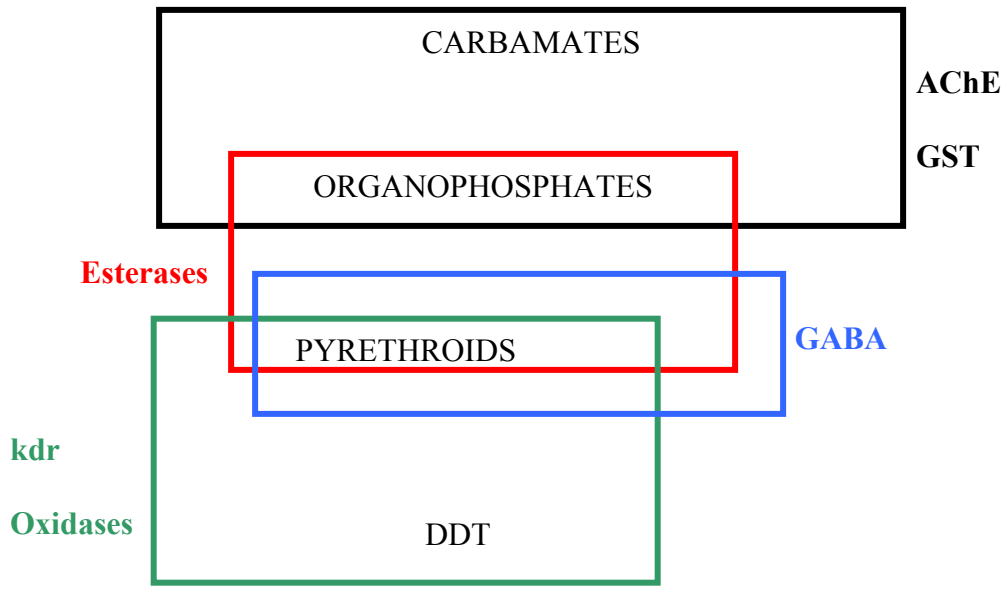


Figure 1.2: Cross-resistance between commonly used insecticides as well as the target site they affect (Adapted from Brogdon and McAllister, 1998).

1.4.1 Target-site insensitivity

Target-site insensitivity resistance involves mutations within the genes targeted by insecticides: sodium-ion channel gene (*kdr*), the γ– aminobutyric acid (GABA) receptor and acetylcholinesterase (AChE) making them less susceptible to insecticides. The knockdown resistance (*kdr)* mutation was first detected in houseflies and the German cockroach (Knipple *et al*., 1994; Williamson *et al*., 1996; Martinez-Torres *et al*., 1997; Liu *et al*., 2000). A point mutation of leucine to phenlyalanine (TTA → TTT) within the S6 hydrophobic transmembrane segment of domain II gives rise to insecticide resistance. Mutation within this region interferes with the binding of pyrethroids or DDT to the voltage-gated sodium channel. This mutation was first detected in *An. gambiae s.s* from West Africa (Martinez-Torres *et al*., 1998). A different mutation (leucine to serine (TTA → TCA)) in the same position was described in *An. gambiae s.s* from East Africa (Ranson *et al*., 2000). Unlike the leucine to phenlyalanine mutation that confers high resistance to both DDT and permethrin, the leucine to serine mutation only confers high resistance to DDT and low resistance to permethrin (Ranson *et al*., 2000).

A second mutation methionine to threonine was identified in the common housefly, *Musca domestica*. This mutation is situated within the loop between the S4 and S5 transmembrane segments of domain II. In association with the leucine/phelyalanine it is referred to as the *super-kdr* mutation (Williamson *et al.,* 1996; Martinez-Torres *et al*., 1997).

Resistance to dieldrin may arise as a result of a mutation of alanine to serine (GCA → TCA) within the *Rdl* (resistance to dieldrin) gene that encodes the γ– aminobutyric acid (GABA) receptor. This mutation was identified in dieldrin resistant *Drosophila* strains (ffrench-Constant *et al.,* 1991; 1993). Investigations carried out on laboratory strains of two dieldrin resistant mosquito species, *An. gambiae* and *An. arabiensis,* determined that two mutations occurred independently. *Anopheles arabiensis* carried the alanine to serine mutation, while *An. gambiae* showed a different mutation of alanine to glycine (Du *et al.,* 2005).

In *Musca domestica* and *Drosophila melanogaster* five mutations in the acetylcholinesterase active site gene were shown to confer resistance to organophosphates and carbamates (Mutero *et al*., 1994; Walsh *et al.,* 2001; Weill *et al.,* 2002). Nabeshima *et al* (2004) reported the encephalitis vector *Culex tritaeniorhynchus* to have an altered AChE (*Ace-2*) gene situated on chromosome 2 associated with organophosphate resistance. Insecticide resistance was also associated with a single mutation in the *Ace-1* gene of *Culex pipiens* (Weill *et al*., 2004).

1.4.2 Metabolic based resistance

Metabolic based resistance is usually brought about by an alteration within the enzyme which in turn affects the detoxification levels and activity (Hemingway *et al.,* 2004). Changes may be qualitative or quantitative (Hemingway and Karunaratne, 1998). Resistance may involve one or more of three major enzyme classes:

carboxylesterases, glutathione-S-transferases and monooxygenases. These enzymes may become involved in the sequestration or detoxification of insecticides. Specific enzyme/s involved in resistance are dependent on the class of insecticide they are able to hydrolyze (Peiris and Hemingway, 1993).

i) Carboxylesterase

Carboxylesterases (also known as esterases) are enzymes that hydrolyze carboxylic esterase (Hemingway and Karunaratne, 1998). There are two forms of esterases, the A and B forms. The B-esterases are inhibited by paraoxon (organophosphate cholinesterase inhibitor) while the A-esterases are not. Esterases are primarily associated with the resistance to organophosphates in a variety of insects. In *Culex* mosquitoes it was found that one or more esterases may be involved in the detoxification of organophosphates. They are also known to hydrolyze carbamates (Hemingway and Karunaratne, 1998). In Sri Lanka, *Culex quinquefasciatus* was reported to have elevated levels of esterases A2 and B2 in a resistant strain which were able to hydrolyze fenitrothion (organophosphate) (Peiris and Hemingway, 1993). High esterase levels were also detected in a population of *An. gambiae* as well as *Aedes aegypti* that was resistant to permethrin (Vulule *et al*., 1999; Flores *et al.,* 2005).

Apart from elevation of the enzyme, amino acid alterations have also been implemented in resistance. An amino acid substitution Gly^{137} to Asp within the active site of both house fly and blowfly esterases conferred resistance to

organophosphates (Claudianos *et al.,* 1999). In the blowfly another substitution Try^{251} to Leu was also reported to confer resistance to organophosphates (Campbell *et al.,* 1998).

ii) Glutathione-S-transferases

Glutathione-S-transferases (GST) are a family of enzymes that are involved in the detoxification and metabolism of endogenous and xenobiotic compounds (Kavishe *et al.*, 2006). Insects have two GST protein families, GST-1 and GST-2 (Fournier *et al.,* 1992). GSTs are dimeric proteins that consist of two binding subunits. A G-site which binds glutathione and an H-site which binds substrate (Hemingway *et al*., 2004). In the housefly, GST-1 is found located within the haemolymph cells while GST-2 is found in the thorax associated with the flight muscles as well as the central nervous system (Franciosa and Bergé, 1995). Increased levels of GST and esterase were detected in Sri Lankan C*ulex tritaeniorhynchus* and *Culex gelidus* resistant to organophosphate, carbamate and pyrethroid insecticides (Karunaratne and Hemingway, 2000). In *An. gambiae,* GSTs have been shown to be involved in DDT resistant mosquitoes (Ranson *et al.,* 2001; Ortelli *et al.,* 2003). Vontas *et al* (2001) detected elevated levels of GST, which conferred pyrethroid resistance in *Nilaparvata lugens* (brown planthopper).

iii) Monooxygenases

Monooxygenase, also known as cytochrome P450, are involved in the oxidation of many endogenous and exogenous compounds (Nelson *et al.,* 1996). They form an

important resistant mechanism for the detoxification of insecticides (Kasai *et al.,* 2000). These enzymes will be discussed in detail in the following sections.

In southern Africa *An. funestus* has been shown to be resistant to pyrethroids (Hargreaves *et al*., 2000; Brooke *et al*., 2001). In biochemical studies, Brooke *et al* (2001) attributed this resistance to elevated levels of monooxygenases. Amenya *et al* (2008) were able to demonstrate an increased expression of a single monooxygenase P450 enzyme associated with resistance. The rest of the thesis will be based on this particular resistance mechanism and will be discussed further in more detail.

1.5 Monooxygenase (Cytochrome P450)

1.5.1 Background

Axelrod (1955) and Brodie *et al* (1955) carried out the first experimental report that suggested an enzyme (Cytochrome P450) was present in the endoplasmic reticulum of rabbit liver that was involved in the oxidation of xenobiotic compounds. In 1958 Garfinkel and Klingenberg separately demonstrated that a pigment within pig and rat liver microsomes was able to bind carbon monoxide and had a distinct absorbent peak at a wavelength of 450 nm (cited from Omura and Sato, 1964a). Owing to its dual properties as an oxidant and reductant this pigment was given the name "mixed function oxidase". Omura and Sato (1964a) specified that Cytochrome P450 was a hemoprotein. They also noted that Cytochrome P450 was associated with another hemoprotein (Cytochrome b_5) within mammalian (rabbit) liver microsomes (Omura and Sato, 1964b).

1.5.2 Nomenclature

Cytochrome P450s are the largest heme-thiolate proteins of a superfamily gene. They are involved in the metabolism of many endogenous (hormones and pheromones) and exogenous (insecticides and plant toxins) compounds (Nelson *et al*., 1996). Owing to the large size of this group of enzymes a naming system has been implemented. The root symbol "CYP" from Cytochrome P450 is placed in front of the family number. For instance those from family 1 are named CYP1. A letter represents a subfamily e.g. CYP1A. Following the subfamily is a number that represents the individual genes present in the specific subfamily e.g. CYP1A1 or CYP1A2 (Nelson *et al*., 1993; Nelson *et al*., 1996; Feyereisen 1999).

1.5.3 Classification

P450s are characterized into four main classes according to the nature of the auxiliary proteins (Miles *et al.,* 2000). Class I P450 proteins are found in most bacteria and embedded in all eukaryotic mitochondrial membranes. This P450 system consists of three components, a flavin adenine dinucleotide (FAD) containing reductase, an iron sulfur protein and the P450 protein/enzyme. Class II P450 system on the other hand only has two components, the NADPH dependent cytochrome P450 reductase consisting of a FAD and flavin mononucleotide (FMN) and the P450 protein (Munro and Lindsay, 1996). Class II is found in eukaryotic microsomes, associated with the endoplasmic reticulum and is involved in the steroid metabolism and detoxification pathways. Class III P450 system is found in bacteria. It is similar to that of class II however the diflavin reductase peptide is fused to that of the P450 protein forming a

single polypeptide (Figure 1.3) (Miles *et al.,* 2000; Werck-Reichhart and Feyereisen, 2000; Roberts *et al.,* 2002). Class IV P450 system was identified by Roberts *et al*. (2002) in bacteria. It consisted of a FMN containing reductase with a ferredoxin- like centre fused to the P450 to form a single polypeptide (Figure 1.3). Table 1.1 illustrates the differences in which the four classes of P450 acquire their electron source.

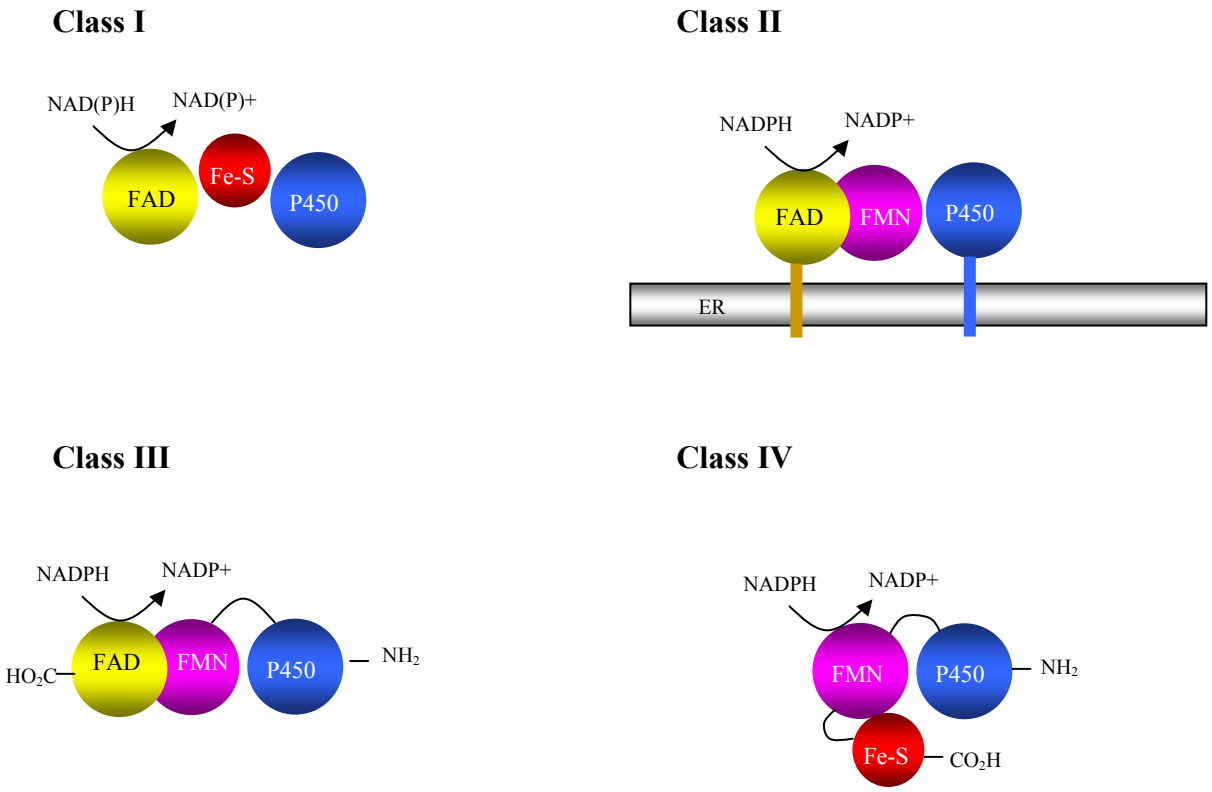


Figure 1.3: Schematic diagrams showing the differences between the four classes of the P450 systems. Class I system comprises of three separate components, FAD-reductase, Fe-S protein and the P450. Class II systems is a membrane bound system composed of a FAD/FMN reductase and the P450. Class III system consists of a FAD/FMN reductase fused to the P450 to make a single polypeptide. Class IV system forms a single polypeptide that is composed of a FMN reductase bound to ferredoxin-like centre fused to the P450. ER-Endoplasmic Reticulum (Adapted from Roberts *et al.,* 2002).

Table 1.1: Classification of Cytochrome P450 (Werck-Reichhart and Feyereisen, 2000)

Classes	Characterization feature
I	Requires an FAD containing P450 reductase and an iron sulfur redoxin
II	Requires only FAD/FMN containing P450 reductase for the transfer of electrons
III	Self sufficient and does not require an electron donor
IV	Receive electrons directly from NAD(P)H

1.5.4 Function and mechanism

Monoxoygenases have diverse enzymatic activities. They are known to occur in a range of functions including steroid biogenetics, xenobiotic detoxification, drug metabolism and many other reactions (Guengerich, 1991). P450s have a general catalytic mechanism as described in Figure 1.4 (Porter and Coon, 1991; Hodgson and Strobel, 1996). The reaction requires electrons from NAD(P)H through redox partners. The oxygen molecule (O_2) is activated which leads to the insertion of one oxygen atom into an organic substrate (RH) and the other is reduced to form water.

$$RH + O_2 + NADPH + H^+ \xrightarrow{P450} ROH + H_2O + NADP^+$$

Figure 1.4: Mechanistic reaction of Cytochrome P450: RH represents substrate.

The reaction is initiated when the substrate binds to the active site of P450. This binding leads to a lowering of the redox potential allowing transfer of an electron from redox partners Cytochrome b_5 and Cytochrome P450 reductase (CPR). Microsomal P450s use Cytochrome P450 reductase that contains FAD and FMN

domains as their electron donor (Degtyarenko and Kulikova, 2001). A change in spin state of the heme iron occurs at the active site. The Fe^{3+} ion is reduced as a result of the first electron transfer (Figure 1.5). Oxygen (O_2) binds rapidly to Fe^{2+} forming Fe^{2+}-O_2. This complex slowly forms a stable Fe^{3+}-O_2^- complex. A second electron transfer occurs, leading to the formation of a superoxide complex (O_2^-). The oxygen (O_2) bond is cleaved resulting in the formation of $(Fe\text{-}O)^{3+}$ complex and water.

The oxygen from Fe-ligand is transferred to the substrate forming a hydroxylated compound. The product is released from the active site and the P450 regains its initial state and the cycle repeats itself (Figure 1.5) (Miles *et al.,* 2000; Munro *et al.,* 2007). Flow of electrons within the P450 system is as follows: NAD(P)H → FAD → FMN → Heme (Smith *et al.,* 1994; Wang *et al.,* 1997). In microsomal P450s the membrane (Phospholipid) has been shown to have an influence on the binding of substrate to P450 as well as the enzyme activity (Murtazina *et al.,* 2004).

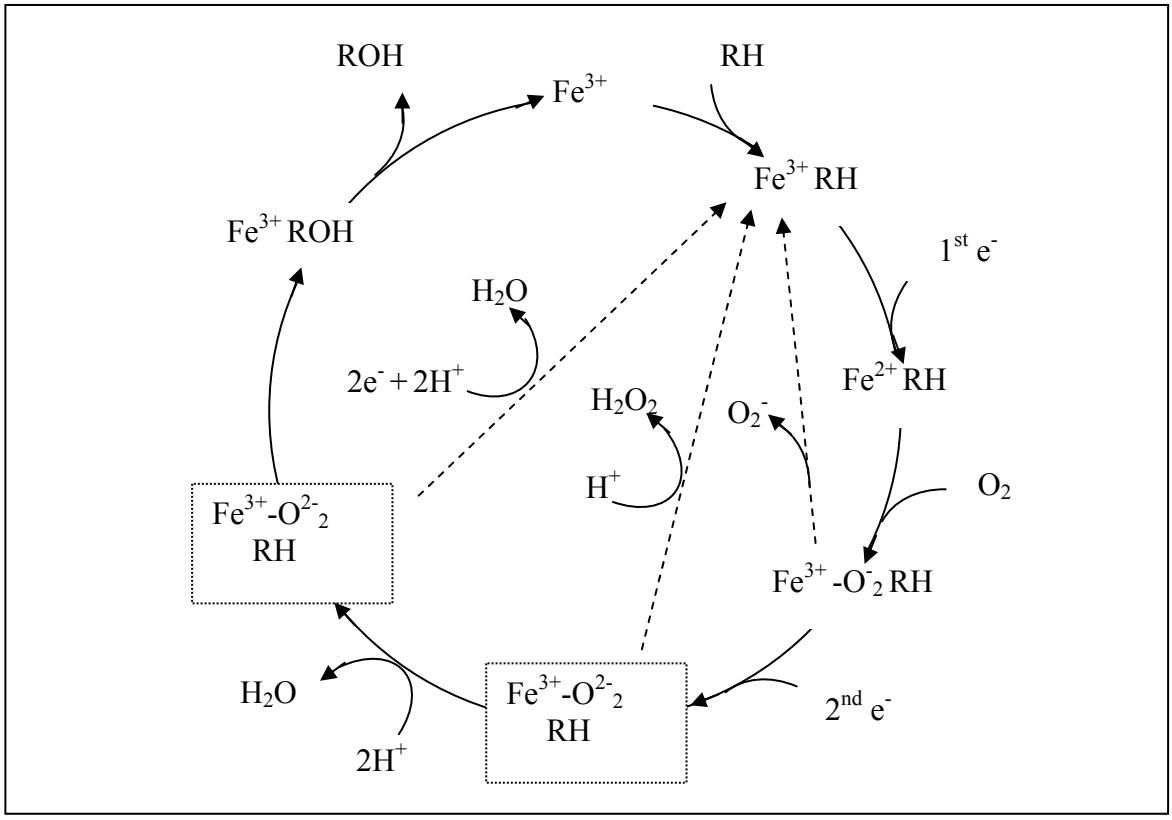


Figure 1.5: Catalytic cycle of P450. RH represents the substrate and ROH is the oxygenated product. (Adapted from Miles *et al.,* 2000; Munro *et al* 2007).

1.5.5 Structure

Poulos *et al* (1985) determined the first crystal structure of *Pseudomonas putida* P450 that was bound to a camphor substrate (P450cam). It was resolved to a resolution of 2.6 Å by x-ray crystallography. The topography of P450 has been shown to have a triangular prim shape (Figure 1.6). Forty percent of the residues make up twelve helical segments. The structure was shown to have five anti-parallel β pairs (Poulos *et al.,* 1985).

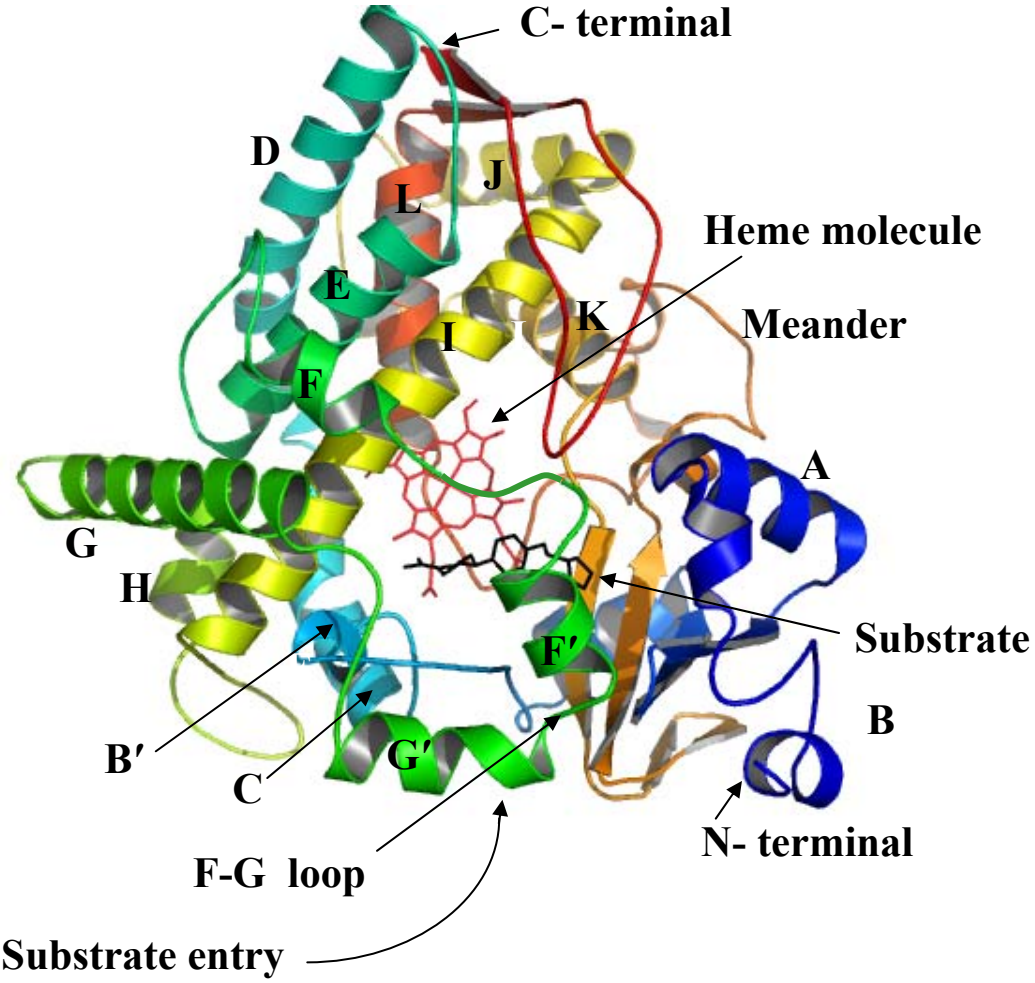


Figure 1.6: Ribbon representation of Cytochrome P450 (human CYP3A4 complexed with ketoconazole). The heme molecule is indicated in red and the substrate in black (PDB code: 2ibjA; Ekroos and Sjögren (2006). A-L indicate helices, C-terminal and N-terminal are indicated. Model was generated using Deep view Swiss-Pdb viewer and PyMOL (Guex and Peitsch, 1997; DeLano, 2002).

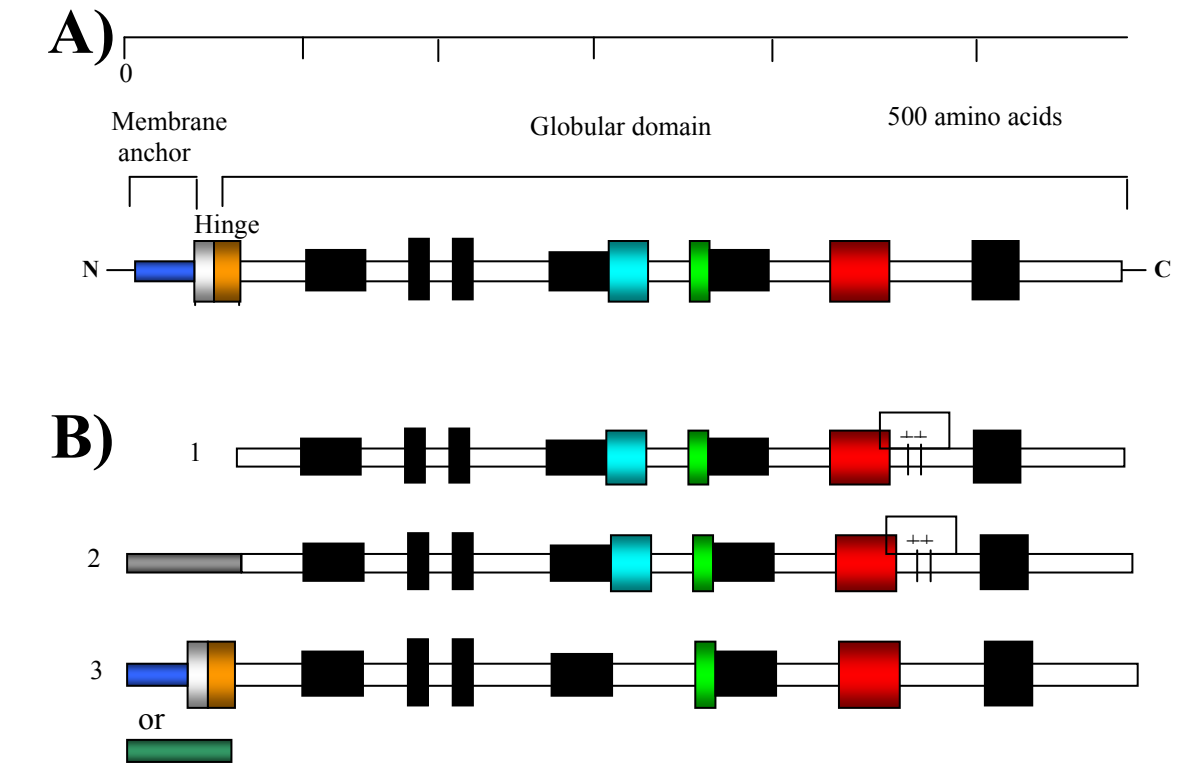
The heme molecule in all P450s is situated within a cavity surrounded by polar residues: four helices (D, E, I, and L) that form a bundle, helices J and K, two sets of β sheets and a coil (meander) (Figure 1.6) (Poulos *et al.,* 1985; Werck-Reichhart and Feyereisen, 2000). Within the cavity on the proximal face of the heme molecule is a highly conserved motif of Pro-Phe-X-X-Gly-X-Arg-X-Cys-X-Gly, which is found near the C-terminus. This motif is characteristic of all P450s and is known to bind the

heme molecule. The heme molecule is stabilized in position by a cysteine residue that forms a fifth ligand to the heme iron (Poulos *et al.,* 1985; Werck-Reichhart and Feyereisen, 2000). The heme cavity structure is stabilized by a conserved motif of Glu-X-X-Arg situated within helix K on the proximal side of the heme molecule. On the distal side of the heme molecule is a proton transfer groove that contains a conserved region (Ala/Gly/-Gly-X-Asp/Glu/-Thr-Thr/Ser) also known as a P450 signature and found on helix I (Werck-Reichhart and Feyereisen, 2000).

Substrates have been shown to bind to six regions within the P450 known as substrate recognition sites (SRSs) (Figure 1.7 A and B). These regions occupy 16% of the total protein (Gotoh, 1992). Amino acid mutations occurring within the SRS regions of P450 family 2 (CYP2) abolished substrate binding (Gotoh, 1992). The substrate binding and recognition sites are the most variable regions of the proteins (Gotoh, 1992; Werck-Reichhart and Feyereisen, 2000). These regions are found near the substrate entry and catalytic sites. They are flexible so as to accommodate the binding and catalytic reaction of substrates (Werck-Reichhart and Feyereisen, 2000). The active site was identified in mammalian CYP2B4 at a resolution of 1.6 Å where a large cleft within the core of the crystal structure was noted. The opening extended from the surface of the protein to the heme molecule. The cleft was formed by helices B' to C and F to G (Figure 1.6) (Scott *et al.,* 2003). In a substrate free form P450cam had a high thermal motion for helices B', F and G suggesting that this region was able to move so as to allow substrates in and out of the active site (Poulos and Johnson, 2005). The F and G helices as well as the joining loop (F-G loop) are

known to be flexible and were identified to operate as a lid in the opening and closing of the active site to allow substrates to enter and leave (Poulos, 2003; Wester, *et al.,* 2004). In order to accommodate substrates helices F and G and the F-G loop have been identified to reshape around the substrate (Yano *et al.,* 2000).

Most eukaryotic P450s are membrane bound and therefore have a hydrophobic N-terminus that anchors into the membrane. This region, like that of the substrate binding and recognition regions, is highly variable. Next to this region is a hinge that consists of Pro-Pro-X-Pro residues that separate the hydrophobic anchor from the rest of the molecule (Figure 1.7) (Werck-Reichhart and Feyereisen, 2000).



Hyper-variable region

-Membrane-targeting hydrophobic segment

-Substrate-recognition site (SRSs)

Conserved features

-Cluster of basic residues

-Cluster of prolines (often PPGP)

-I-helix groove: distal face of the heme involved in catalysis

-Glu-X-X-Arg mortif in the K helix

-Heme-binding loop including cysteine coordinated to heme iron

Features specific to non-ER proteins

-Two positive charges (interaction with ferredoxin)

-Mitochondrial
-Chloroplast
} targeting sequence

Figure 1.7: Primary and tertiary structure of Cytochrome P450. A) Structure of an ER-bound Cytochrome P450 protein. B) Structure of various P450 proteins, 1: soluble class I, 2: Mitochondrial class II, 3: membrane–bound class III. Key shows the various functions of the colour-coded domains (Adapted from Werck-Reichhart and Feyereisen, 2000).

1.5.6 P450 redox partners; Cytochrome P450 reductase and Cytochrome b_5

Cytochrome P450 reductase and cytochrome b_5 are important in the P450 system as they are the source of electron donors from NADPH to microsomal P450 proteins. Without redox partners microsomal P450 enzymes are unable to function (Degtyarenko and Kulikova, 2001).

1.5.6.1 Cytochrome P450 reductase (CPR)

Microsomal NADPH-cytochrome P450 reductase (CPR) (~77 kDa) are membrane bound flavoproteins that contain FAD (similar to flavodoxin) and FMN/NADPH (similar to ferredoxin-$NADP^+$ reductase) binding domains. The x-ray crystal structure of the CPR was resolved at 2.6 Å. The FMN domain is situated at the N-terminus and contains a hydrophobic membrane anchor region. The FAD and NADPH binding domains are separated from the FMN domain by a connecting region (Figure 1.8) (Wang *et al*., 1997). CPR is involved in the catalytic transfer of the first electron from NADPH. Electrons are accepted from NADPH by the FAD which in turn transfers them to FMN that is interacting with the P450 resulting in the reduction of P450 (Sevrioukova *et al.,* 1999b; Smith *et al*., 1994; Wang *et al*., 1997). Binding of eukaryote microsomal P450 to CPR was resolved at a resolution of 2.03 Å. The flavin domain was found on the proximal side of the P450 facing the heme molecule (Sevrioukova *et al.,* 1999b). Bridges *et al* (1998) carried out two mutations on the proximal surface of P450 that resulted in a decrease in binding affinity for CPR.

Non-membrane bound CPR do not efficiently interact with P450 during electron transfer. Anchoring of the CPR to a membrane creates a suitable orientation for efficient transfer of electrons to P450. CPR is embedded within the membrane in such a way that the FMN and FAD/NADPH domains are close to the membrane surface so as to have the FMN and P450 in close proximity (Paine *et al.,* 2005). The membrane (Phospholipid) plays an important role in the interaction between P450 and CPR complex (Müller-Enoch *et al.,* 1984; Paine *et al.,* 2005).

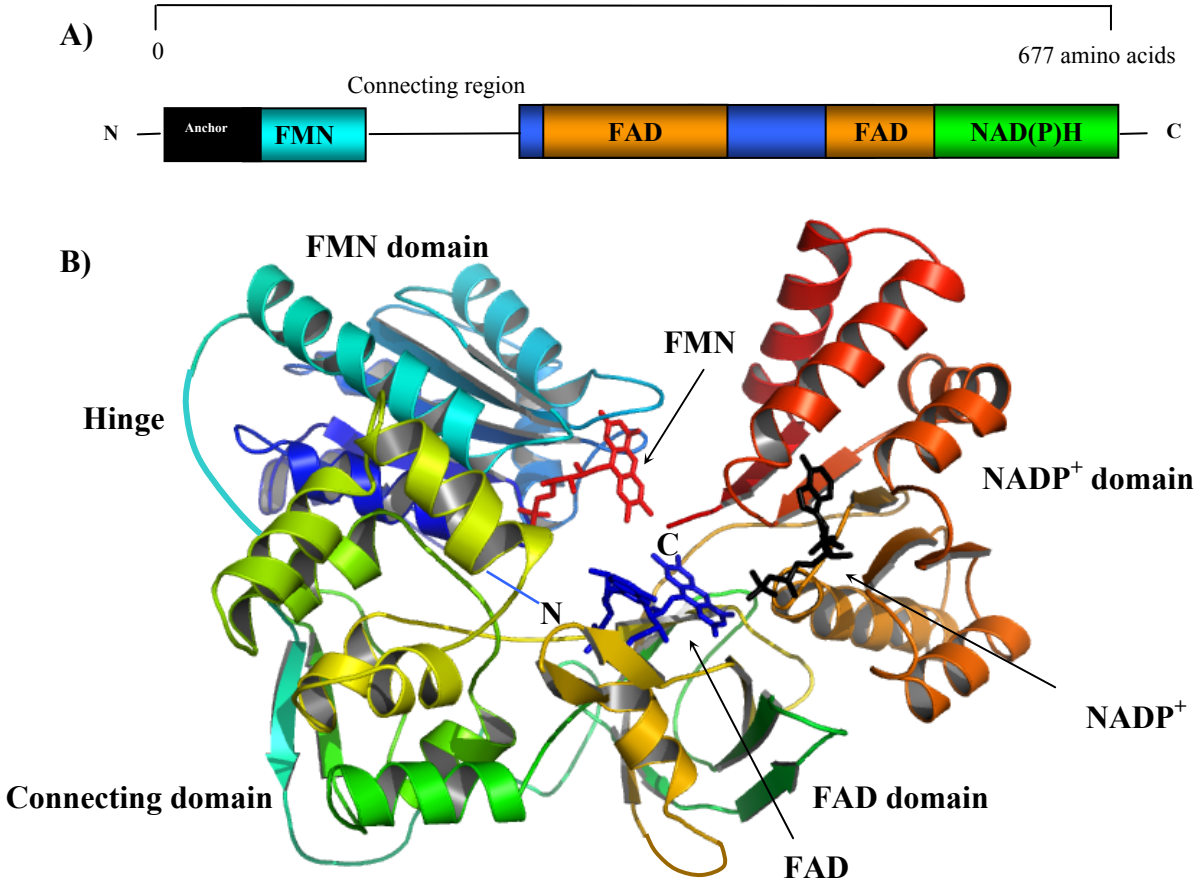


Figure 1.8: Ribbon representation of Cytochrome P450 reducatse (CPR). A) Liner representation of the CPR showing the anchor (Black), FMN (Blue), FAD (Orange) and NAD(P)H (Green) domains (Adapted from Wang *et al*., 1997). B) Ribbon representation of the tertiary structure of CPR. The cofactors FMN (Red), FAD (Blue) and $NADP^+$ (Black) are shown in position. N: N-terminal, C: C-terminal. (PDB code: 1amoA: Wang *et al*., 1997) Model was generated using Deep view Swiss-Pdb viewer and PyMOL (Guex and Peitsch, 1997; DeLano, 2002).

1.5.6.2 Cytochrome b_5 (cytb_5)

Cytochrome b_5 (cytb_5) is a heme containing protein that consists of about 140 amino acids (making up a ~17 kDa protein). The crystal structure has been resolved at 2.8 Å (Argos and Mathews, 1975). Cytb_5 are membrane-bound globular proteins that are found within microsomes. They consist of a heme group situated within a hydrophobic pocket that is held in position by two histidine residues (Figure 1.9) (Rogers and Strittmatter, 1973; Argos and Mathews, 1975). The second electron within the P450 reaction has been predicted to come from cytb_5. The redox potential of cytb_5 is +25 mV while P450 is -300 mV. This suggests that cytb_5 may only be able to donate the second electron rather than the first (Paine *et al.,* 2005). In the rabbit CYP2B4 system, cytb_5 has been shown to transfer electrons faster than CPR (Gruenke *et al*., 1995). In solution cytb_5 has two isomer forms, the A and B forms, that differ from each other at a rotation of 180º along the heme plane around the α and γ meso protons (Arnesano *et al*., 1999). Cytb_5 have been shown to bind to P450 on the proximal surface of the P450 heme domain (Bridges *et al*., 1998; Sevrioukova *et al*., 1999a; Sevrioukova *et al*., 1999b). Seven mutations on the proximal surface of P450 showed a decrease in binding affinity with cytb_5 (Bridges *et al*., 1998). The mechanism of electron transfer from cytb_5 to P450 is not clearly understood. There have been suggestions that electrons flow from NADPH → CPR → cytb_5 → P450 or NADPH → cytb_5 reductase → cytb_5 → P450 (Paine *et al.,* 2005).

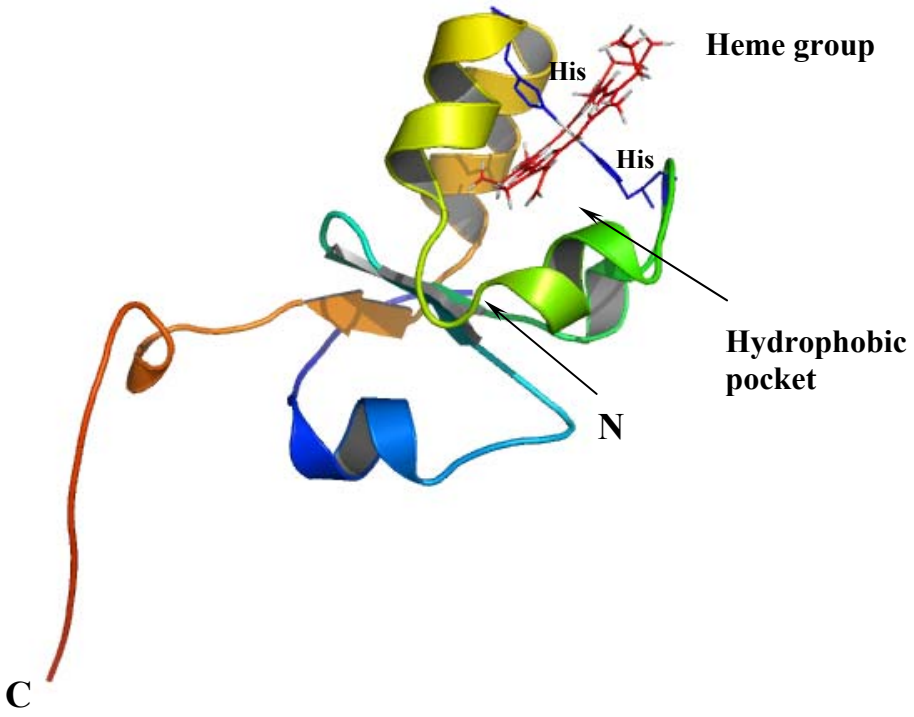


Figure 1.9: Ribbon representation of cytochrome b_5 (cytb_5). The heme group (red) is situated within a cleft surrounded by hydrophobic residues (hydrophobic pocket). The two histidines are indicated in blue. (PDB code: 1do9A; Banci *et al*., 2000). N: N-terminal, C: C-terminal. Model was generated using Deep view Swiss-Pdb viewer and PyMOL (Guex and Peitsch, 1997; DeLano, 2002).

1.6 Insect Cytochrome P450 and insecticide resistance

P450 genes are grouped into clans that are derived from a common ancestor. The clans are named according to the lowest family in the clan (Nelson, 1999). Insect P450 have four clades which are i) CYP2 clade, these are P450s that are involved in the physiological function and those that have broader substrate specificity, ii) CYP3 clade, these are the insect CYP6 genes which were the first to be cloned and characterized within this clade they are closely related to the vertebrate CYP3 and CYP15 families. Insect P450 genes in this clade are the most numerous and are mainly found in large clusters. Members in this clade are associated with xenobiotic

metabolism as well as insecticide resistance an example is CYP6Z1 that is overexpressed in pyrethroid resistant *An. gambiae,* iii) CYP4 clade, genes from this clade are numerous in insects. They are involved in a diversity of functions and are inducible metabolizers of xenobiotics such as CYP4C27 in *An. gambiae* that is overexpressed in DDT resistant strain, iv) Mitochondrial clades: in insects there are two types one that serves as a physiological function and those that are taxon specific paralogous P450s. In *Drosophila* and *Anopheles* CYP12 belong to this clade and are associated with insecticide resistance (Claudianos *et al.,* 2006; Feyereisen, 2006).

The majority of P450 studies have been carried out in mammalian systems. It was determined however that the structure and mechanisms were conserved from prokaryotes (bacteria) to eukaryotes (*Drosophila melanogaster*) (Feyereisen, 1999). In insects P450s are found within all tissues of the insect (Feyereisen, 1999). P450 enzymes play an important role in the detoxification of insecticides therefore conferring resistance (Bergé *et al*., 1998; Feyereisen, 1999; Scott, 1999; Hemingway and Ranson, 2000). They have been implicated in insecticide resistance mechanisms in a number of insect species. For instance, in *Drosophila melanogaster* it was found that three substitution mutations in the P450 gene CYP6A2 conferred resistance. These mutations were found on the dorsal side of helix I within the vicinity of the substrate recognition valley of the active site. This region is important for enzyme activity (Bergé *et al*., 1998; Amichot *et al*., 2004).

Rongnoparut *et al* (2003) reported two novel P450 genes belonging to the CYP6 family (CYP6AA2 and CYP6P5). In their findings they noted that CYP6AA2 mRNA transcription was produced at a greater amount compared to that of CYP6P5 when carrying out mRNA expression studies on both resistant and susceptible mosquitoes. It was therefore concluded that CYP6AA2 was involved in resistance. Pyrethroid resistant houseflies were noted to have a high transcription level of CYP6D1 mRNA, indicating that CYP6D1 was involved in pyrethroid resistance (Kasai and Scott, 2000). The CYP6 family has been implicated in insecticide resistance more often than any other CYP family to date.

Apart from the CYP6 family, three other CYP families (CYP4, CYP9 and CYP12) have also been implicated in insecticide resistance (Danileson *et al*., 1997; Guzov *et al*., 1998; Hemingway and Ranson, 2000; Scharf *et al*., 2001; Ranson *et al*., 2002a). In studies carried out on western corn rootworms it was noted that mRNA transcript signals from CYP4 genes (CYP4AJ1, CYP4G18 and CYP4AK1) were increased in insecticide resistant individuals speculating that CYP4 are involved in organophosphate resistance (Scharf *et al.,* 2001).

In the tobacco budworm, *Helithis virescens* CYP9A1 was the first member of the CYP9 family to be isolated associated with carbamate resistance. It was found to be similar to the insect CYP6 family (Rose *et al.,* 1997). Reports on housefly CYP12 showed high levels of CYP12A1 mRNA in insecticide resistant flies. CYP12A1 was

expressed and purified from *Escherichia coli* and was able to metabolize a variety of insecticides as well as some xenobiotics (Guzov *et al*., 1998).

In order to characterize genes differentially expressed in resistant *An. gambiae*, David *et al* (2005) developed a microarry detoxification chip. This array was able to isolate five strongly up-regulated genes present in DDT resistant *An. gambiae*. Amongst these five genes, two were P450s. Of these, CYP6Z1 was previously isolated and shown to have a high expression level in resistant mosquitoes (Nikou *et al.,* 2003). Up-regulation in the other (CYP325A3) was unexpected as it had never previously been implicated in insecticide resistance. Plant alkaloid detoxification P450s, CYP4D1 and CYP4D10 of *Drosophila melanogaster* and *Drosophila mettleri* respectively were shown to have high amino acid sequence similarity to that of CYP325A3. It is therefore possible that this enzyme might also be involved in detoxification.

The *An. gambiae* genome contains 111, P450 genes of which seven are pseudo genes (Ranson *et al.,* 2002b). Only 31, P450 genes have been identified from *An. funestus* (Amenya *et al.,* 2005). Amenya *et al* (2005) identified 12 CYP4, 12 CYP6 and 7 CYP9 partial genes that have a high percentage sequence similarity to that of *An. gambiae*. Only one of these was unique to *An. funestus* (CYP9J14) and showed a low similarity (55%) to any known *An. gambiae* CYP9 ortholog. Amenya *et al* (2008) have further shown that one of these CYP6P9 is overexpressed in a pyrethroid resistant *An. funestus* laboratory colony.

1.7 Hypothesis

Cytochrome P450 (CYP6P9) is involved in the protection of *An. funestus* exposed to pyrethroid insecticides. Its resistance mode of action is detoxification by hydrolysis of pyrethroids.

1.8 Objective

The objective of this study is to isolate the full length coding region of CYP6P9 from pyrethroid resistant (FUMOZ-R) and susceptible (FANG) strains. Compare deduced amino acid sequences. Cytochrome P450 heterologous expression systems will be investigated to obtain high levels of CYP6P9. This will be used in metabolic studies to determine if cytochrome P450s in the resistant stain are able to hydrolyze pyrethroids (permethrin) to its metabolites. Redox partners (CPR and cytb_5) will be isolated so as to co-express with CYP6P9 to obtain a functional P450 system.

1.9 Approach

To achieve the objectives a number of approaches were carried out. These involved isolation and identification of CYP6P9 from *An. funestus* pyrethroid resistant (FUMOZ-R) and susceptible (FANG) strains. BLAST (Basic Local Alignment Search Tool) was used to determine if the correct genes (belonging to cytochrome P450, CYP6 family) were isolated. Cytochrome P450 redox partners (cytochrome P450 reductase and cytochrome b_5) from *An. funestus* were also isolated and confirmed by BLAST. Bioinformatic tools such as multiple sequence alignment was used to identify deduced amino acid differences between FUMOZ-R and FANG

CYP6P9. Helices and conserved domains were also identified. A three-dimensional structure was generated using homology modeling to visualize structural differences between FUMOZ-R and FANG CYP6P9.

Cytochrome P450 expression systems have never been performed in *An. funestus*. We therefore analyzed various techniques. In *Escherichia coli (E. coli)* recombinant proteins were expressed using the insect pIX4.0 plasmid and pCWori+ plasmid. Two techniques were employed; i) Bacterial leader sequences *ompA* (Outer Membrane Protein A) was attached to the hydrophobic NH_2-terminal of CYP6P9 to allow for the expression of the full length CYP6P9 in *E. coli* host without modifying the NH_2-terminus hydrophobic region. ii) Modification of the NH_2-terminus by replacing the first eight amino acid sequences with a short bovine steroid 17 α-hydroxylase (MALLLAVF) amino acids sequence.

Purification was performed using a 6x His tag purification system employing the Ni^{2+}-chelating affinity chromatography. High Performance Liquid Chromatography (HPLC) and Mass Spectroscopy (MS) were used to determine metabolic activity of pyrethroids (permethrin) by P450.

Chapter Two

Isolation and characterization of *Anopheles funestus* total microsomal cytochrome P450

2.1 Introduction

2.1.1 Cytochrome P450 enzyme activities

Cytochrome P450s found within the membrane of microsomes are referred to as microsomal P450s and acquire electrons via electron donors (CPR and cytb_5) (Yamazaki *et al.,* 1999a). In the presence of electron donors microsomal P450s are known to play important roles in the metabolism of many xenobiotics and endogenous compounds. These enzymes are cable of catalyzing several substrates and can overlap in substrate specificity (Korytko *et al.,* 2000; Brown *et al.,* 2003).

P450 enzymes are a major cause of insecticide resistance in insects. In the house fly, CYP6A1 and CYP12A1 were shown to metabolize a variety of insecticides such as Aldrin, Heptachlor, Diazinon, and Azinphosmethyl, (Guzov *et al.,* 1998; Sabourault *et al.,* 2001). A number of substrate models such as benzo[a]-pyrene, *p*-chloro-*N*-methylaniline, *p*-nitroanisole, and 7-ethoxycoumarin have been designed to test the activity of P450. Ethoxycoumarin deethylase (ECOD) activity is a widely used P450 assay which acts as an indicator for oxidative metabolism (Frank, *et al.,* 1997). Amichot *et al* (2004) also demonstrated that CYP6A2 from fruit flies were able to

metabolise DDT to dicofol, dichlorodiphenyl-dichloroethane (DDD) and dichlorodiphenyl acetic acid (DDA).

2.2 Objective

Enzyme activity determines the efficiency at which the enzyme is able of catalysing substrates. This is important in insecticide resistance as this relates to the extent to which insecticides are hydrolysed by particular enzymes. Metabolites produced from insecticide metabolism further supports the role of the enzyme in detoxification resistance. The broad objective of this study was to compare the enzyme activity of total microsomal P450 from pyrethroid resistant and susceptible *An. funestus* using 7-ethoxycoumarin as substrate. Total microsomal P450 activity from *An. funestus* was also compared with those of other insect P450 activity. Permethrin metabolism was conducted using total microsomal P450 from both resistant and susceptible strains. The products were analyzed on a reverse phase HPLC.

2.3 Materials and Methods

2.3.1 Mosquito strains

Two strains of *Anopheles funestus* colonized at the National Institute for Communicable Diseases (NICD) in Johannesburg, South Africa, were used. The pyrethroid resistant strain (FUMOZ-R) is derived from material collected in southern Mozambique and the insecticide susceptible strain (FANG) is derived from material collected in southern Angola. Both strains are kept under standard insectary conditions (25 ºC with a relative humidity of 80% and a 12 hour day/night light). Selection of pyrethroid resistance in FUMOZ-R was performed by separating newly emerged male and female adults into cages supplied with 10% sucrose solution. This ensured that mating did not occur prior exposure so as to select resistant strains. A standard WHO bioassay was carried out by exposing 2-5 day old adults for one hour on filter papers treated with 0.1% lamba-cyhalothrin. Exposure tubes contained about 25 adults per exposure. A total of about ten repeats were performed per age for each generation. Survivors after 24 hour post-exposure from both male and female were allowed to mate over a period of six days. Females were supplied daily with blood meals in order to produce eggs. Eggs were reared to adults and resistance to lamba-cyhalothrin was performed as above (Hunt *et al*., 2005). Selection for permethrin resistance was performed as above on resistant adults using 1.5% permethrin. Once resistance was selected the dosage was reduced to the standard 0.75% permethrin using filter papers supplied by WHO.

2.3.2 Determination of permethrin (pyrethroid) resistance

Resistance to permethrin was performed in order to confirm the level of resistance in FUMOZ-R compared to susceptible FANG strain. Three day old mosquitoes (n=25) were transferred into a standard WHO holding tube (tubes containing untreated filter paper) (Figure 2.1). The mosquitoes were then transferred into exposure tubes (containing 0.75% permethrin paper) and exposed for 1 hour after which the knockdown rate was recorded. The mosquitoes were transferred back into holding tubes and supplied with 10% sucrose solution (soaked in a cotton wool pad) and left for 24 hours (Figure 2.1). A final mortality was recorded after 24 hours. Three replicates were performed (WHO, 1998).

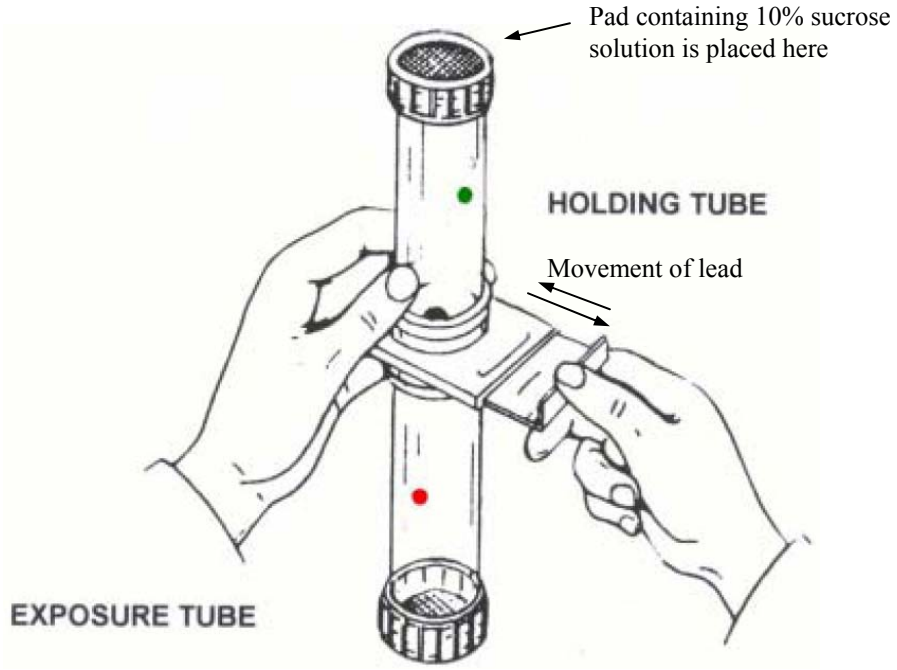


Figure 2.1: Standard WHO holding tube. Mosquitoes are first transferred into the holding tube. They are then transferred to the exposure tube containing permethrin treated paper by opening a lead that separates the two tubes. After one hour exposure mosquitoes are transferred back to holding tube for 24 hours and supplied with a pad containing 10% sucrose solution (WHO, 1998).

2.3.3 P450 inhibition

Synergist bioassays on FUMOZ-R and FANG strains

Synergists are used to inhibit/block the function of P450 activity. The assay was performed on both resistant and susceptible strains in order to confirm the involvement of P450 in permethrin resistance. Synergist papers were prepared by cutting 12 X 15 cm Whatman no. 1 filter papers. A 4% solution of Piperonyl butoxide (PBO) (0.7 ml [4% PBO in olive oil] and 1.2 ml acetone) was pipetted evenly onto the filter paper. A lead pencil was used to mark the treated surface and papers were allowed to dry in a fume hood for 24 hours (developed from Hemingway PhD thesis, 1981).

Mosquitoes (n=25) were transferred into a standard WHO exposure tube as previously described in section 2.3.2. Mosquitoes were exposed to the synergist (4% PBO) for 1 hour before transferring to a tube containing permethrin (0.75%) paper for a further 1 hour with 5minute interval recording of knockdown. The mosquitoes were then transferred into holding tubes and supplied with 10% sucrose solution and left for 24 hours. A final mortality was recorded after 24 hours. Controls included exposure to 4% PBO only and to 0.75% permethrin only.

2.3.4 Preparation of microsomes

Microsomes were prepared from adult mosquitoes (n=100) and larval midguts (n=100) which were homogenised in a cold mortar and pestle that was stored at -80 ºC. The mosquitoes or midguts were homogenised in liquid nitrogen to form a

powder. Buffer A (1 ml; 0.1 M Na_2HPO_4, 1 mM EDTA, 1mM DTT, 200 mM sucrose, 1 µg/ml aprotinin, 1 µg/ml leupeptin and 1 mM PMSF) was added to the homogenised midguts/mosquitoes and mixed. Sample was centrifuged at 5,000 *x g* for 5 minutes and supernatant collected. The supernatant was centrifuged for 20 minutes at 10,000 *x g*. The supernatant was collected and centrifuged at 50,000 *x g* for 1 hour. The pellet was collected and Buffer # A added (modified from Perry and Bucknor, 1970). Total protein content was measured using the Bradford's reagent and BSA as the standard (Table 2.1). Absorbance reading was measured at 595 nm.

Table 2.1: Protein determination by Bradford's assay (standard curve)

BSA(2 mg/ml) (Volume, µl)	µg/µl	Bradford'sreagent (20%) (Volume, µl)
0	0	300
1	2	300
2	4	300
4	8	300
5	10	300
6	12	300
8	16	300

Bradford's reagent (300 µl) was added to microsomal P450 samples (2 µl) in a microtitre plate.

2.3.5 P450 Assay

i) 7-hydroxycoumarin standard curve

Substrate 7-ethoxycoumarin was used to assay P450 activity. In the presence of P450 enzyme, 7-ethoxycoumarin is hydrolysed to 7-hydroxycoumarin. A standard curve

was prepared using 7-hydroxycoumarin. Sodium phosphate buffer pH 7.2 (0.1 M Na_2HPO_4; 1 mM EDTA; 1 mM DTT; 200 mM Sucrose) (Buffer A) was added to various concentrations of 7-hydroxycoumarin (Table 2.2). Readings were measured in a fluorospectrophotometer (Varioskan ™, Thermo Electron Corporation, Finland) at an emission of 465 nm and excitation of 390 nm and plotted on a graph against concentrations of 7-hydroxycoumarin.

Table 2.2: 7-hydroxycoumarin Standard assay

nmol	7-hydroxycoumarin (µM)	Buffer A
0	0	100
5	5	95
10	10	90
20	20	80
40	40	60
60	60	40
80	80	20
100	100	0

ii) P450 assay on An. funestus microsomal P450.

In a microtitre plate (white walled) total microsomal P450 (50 µl) was added. Mix A (50 µl; Buffer A, 33.5 µl; 20 mM 7-ethoxycoumarin, 2.5 µl; 14 µl of 10 mM NADPH) was added to each well to last for the duration of the reaction. The reaction was incubated for 30 minutes at 30 °C. Mix B (22.5 µl; 30 mM Glutathione Oxidized (GS-SG), 10 µl; 0.5 units Glutathione reductase, 0.5 µl; water, 12 µl) was added and allowed to incubate for 10 minutes at room temperature. The reaction was stopped with Tris-acetonitrile (27, 5 µl) (50 mM Tris pH 8; 1:1 acetonitrile). Readings were

measured in a fluorospectrophotometer at an emission of 465 nm and excitation of 390 nm. Controls included reactions with no NADPH and another with no enzyme (buffer only).

2.3.6 Permethrin metabolism

Total microsomal P450 (50 µl) was mixed as in Table 2.3. The reaction was incubated in the dark for 2 ½ hours. A sample (50 µl) was removed and the reaction stopped with acetonitrile (50 µl) and centrifuged at 16,000 *x g* for 3 minutes.

Table 2.3 Reaction mixture for permethrin detoxification

Component	Stock	Reaction concentration	Volume, µl
$MgCl_2$	10 mM	1 mM	10
Glucose-6- phosphate	100 mM	4 mM	4
NADP+	10 mM	1 mM	10
G6P dehydrogenase*	38.5 U/ml	1 U/ml	2.6
K_2HPO_4 pH 7.4	500 mM	50 mM	10
Microsomal P450			50
Permethrin	5 mM	0.05 mM	1
Water			12.4
Volume			100

*G6P dehydrogenase (glucose -6-phosphate dehydrogenase)

The supernatant was loaded onto an HPLC (Ultimate 3000, Dionex, UK) with a flow rate of 1.0 ml /min and detected at 232 nm over a period of 30 minutes. Distinctive peaks from test samples (FUMOZ-R and FANG) were compared with controls that had no enzymes (No microsomal P450).

2.3.7 Permethrin extraction from adult mosquito

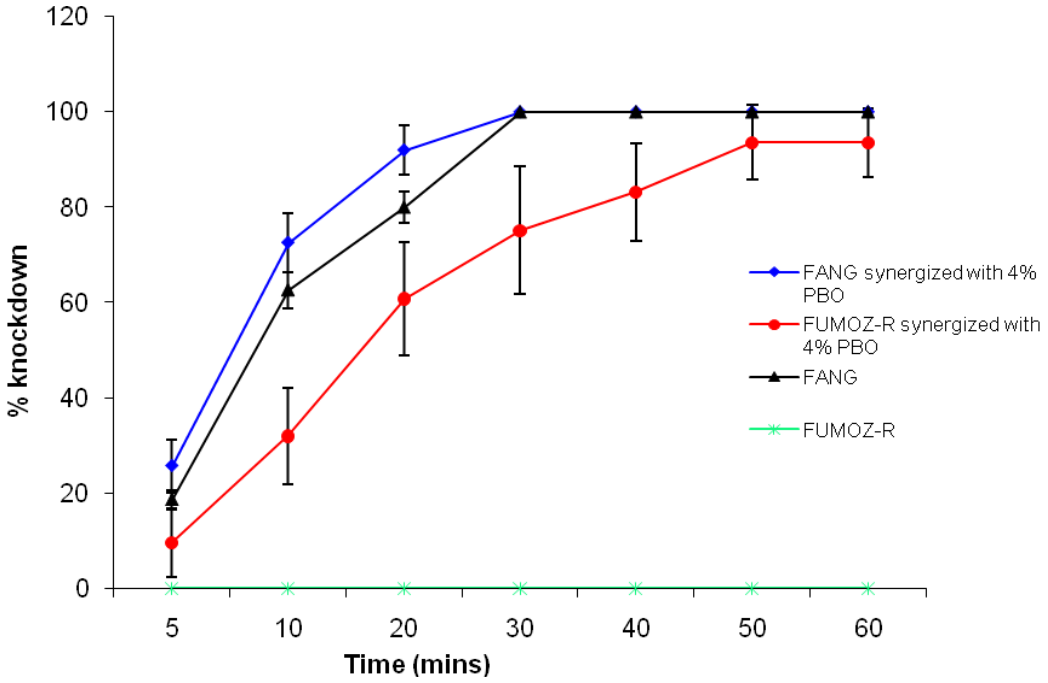
Permethrin was extracted from adult mosquitoes previously exposed to 0.75% permethrin in order to isolate metabolites produced as a result of detoxification. Extraction was performed on three sets of mosquitoes: exposure to 0.75% permethrin for one hour, PBO treated and then exposed to permethrin for one hour and 20% knockdown (mosquitoes exposed to 0.75% permethrin until 20% knockdown is achieved). Each mosquito was dipped in methanol to rinse off any excess permethrin attached to the outer body as a result of exposure. The mosquito was dried on filter paper. Mosquitoes (n=50) were homogenised in sodium phosphate buffer pH 7.2 (0.1 M Na_2HPO_4; 1 mM EDTA; 1 mM DTT; 200 mM Sucrose). The homogenate was transferred to a glass tube (tube A) and chloroform (2 ml) was added. Chloroform slowly dissolves plastic tubes hence the use of glass tubes. The mix was centrifuged at 3,000 *x g* for 5 minutes. The lower organic layer was removed using a glass pipette and placed into a clean glass tube (tube B). Sulphuric acid (2 drops) was added to tube A containing homogenate followed by the addition of chloroform (2 ml). The tube was centrifuged at 3,000 *x g* for 5 minutes and the lower organic layer transferred to tube B. Tubes were placed in a fume hood and chloroform was evaporated by passing nitrogen gas over extraction. The pellet was dissolved in 200 µl of acetonitrile. The sample was loaded onto an HPLC (Ultimate 3000, Dionex, UK) with a flow rate of 1.0 ml /min and a wavelength of 232 nm over a period of 30 minutes.

2.4 Results

2.4.1 Determination of pyrethroid resistance and P450 inhibition

The role of P450s in resistance was analyzed by carrying out two tests: the permethrin resistance and the P450 synergist assays. Resistant (FUMOZ-R) and susceptible (FANG) strains were exposed to permethrin (0.75%) for one hour. FUMOZ-R obtained a zero percent knockdown while 100% knockdown was observed for FANG after 60 minutes exposure (Figure 2.2A). A synergist bioassay for P450 inhibition using 4% Piperonyl butoxide (PBO) was performed on both FUMOZ-R and FANG strains. Mosquitoes were exposed to PBO for one hour followed by a further one hour exposure to 0.75% permethrin during which knockdown was measured. After 30 minutes exposure, FANG had 100% knockdown while FUMOZ-R had a knockdown average of 93% (Figure 2.2A). Mortality rate was measured after 24 hours post exposure. FUMOZ-R (exposed to 0.75% permethrin only) had a zero percent mortality while FANG had 100% mortality (Figure 2.2B). Samples treated with PBO then exposed to 0.75% permethrin (FUMOZ-R synergized and FANG synergized) had 100% mortality 24 hours post exposure (Figure 2.2B). A 20% knockdown was achieved when FUMOZ-R was exposed for three hours to permethrin (0.75%). FANG only obtained 20% knockdown after three minutes exposure to permethrin (0.75%).

A)



B)

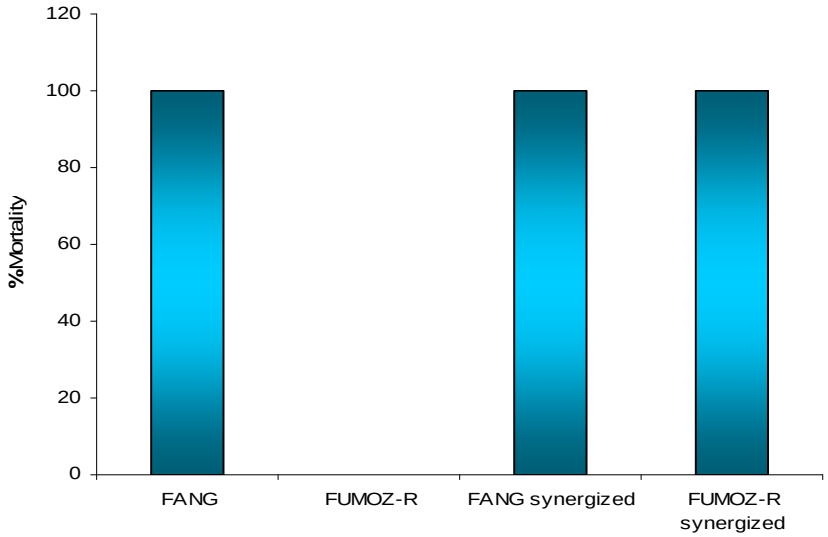
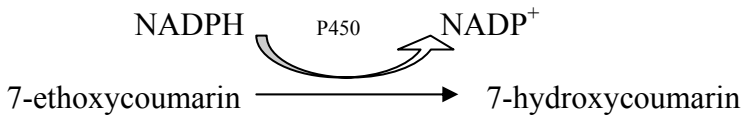


Figure 2.2: Exposure to 0.75% permethrin and P450 synergist using 4% piperonyl butoxide. A) Mosquitoes (FUMOZ-R and FANG) were exposed for 1 hour to 0.75% permethrin. Synergist was performed by exposing for 1 hour to 4% PBO, and then exposed to 0.75% permethrin for a further 1 hour during which knockdown was measures. B) Percentage mortality after 24 hours post exposure with 0.75% permethrin. Standard deviation is represented by error bars.

2.4.2 Total microsomal P450 enzyme assay

Total microsomal P450 were isolated from midguts of mosquito larvae (4th instar) and headless adult mosquitoes. Heads were removed from adult mosquitoes as a result of the eye pigment (xanthommatin) which is a P450 inhibitor (Danielson and Fogleman, 1994). Cytochrome P450 assay using 7-ethoxycoumarin was performed on larvae midguts and adult mosquitoes to investigate and compare the levels of P450 activity. Total protein content of P450 was determined using the Bradford's standard curve (Appendix A2). The P450 spectrum generally used to measure P450 activity was not used due to insufficient P450 protein isolated from microsomal. In the presence of P450, 7-ethoxycoumarin is hydrolysed to 7-hydroxycoumarin as seen below. Hydrolysis of 7-ethoxycoumarin to 7-hydroxycoumarin was determined using the 7- hydroxycoumarin standard curve (Appendix A3).



FUMOZ-R had a relatively higher P450 activity for both larval midgut and headless adult than that of FANG. Although other proteins such as GSTs are found within the microsome fraction, the assay is specific to only detect P450s. The highest total microsomal P450 activity was measured in larval midgut of FUMOZ-R (4.127 ± 1.02 nmol/min/mg P450 protein). FUMOZ-R headless adult had a slightly higher activity (1.573 ± 0.58 nmol/min/mg P450 protein) than the larval midgut of FANG (1.134 ± 0.58 nmol/min/mg P450 protein). The lowest activity was measured with FANG

headless adult (0.247 ± 0.09 nmol/min/mg P450 protein) (Figure 2.3). Statistical analysis reveled that there was a significant difference (0.0116) between the larval midgut P450 activity of FUMOZ-R and FANG. P450 analysis between headless adults of FUMOZ-R and FANG also showed a significant difference (0.0177) using the *t*-test, $p < 0.05$ (Statistix 7, Analytical software version 7.0).

Mosquitoes that were exposed to 0.75% permethrin, 20% knockdown and P450 synergized with PBO were also assayed. Permethrin exposure was carried out in order to induce P450 enzymes. If induction occurred then high P450 activity would be expected. PBO treated mosquitoes served as controls and low or no P450 activities were expected. FUMOZ-R again had the highest activity. FUMOZ-R (20% knockdown) which was exposed to 0.75% permethrin for three hours had the highest activity (1.036 ± 0.91 nmol/min/mg P450 protein). Those that were 1 – hour exposed (FUMOZ-R) had a slightly lower activity (0.999 ± 0.19 nmol/min/mg P450 protein) than the 20% knockdown (FUMOZ-R). FANG, exposed (1hour exposure) (0.374 ± 0.13 nmol/min/mg P450 protein) had a relatively low activity compared to that of FUMOZ-R. Both strains which were treated with PBO had low activities (0.254 ± 0.11 and 0.0911 ± 0.14 nmol/min/mg P450 protein for FUMOZ-R and FANG respectively) indicating that the total P450s were inhibited with PBO. The lowest activity was obtained from 20% knockdown FANG (0.016 ± 0.19 nmol/min/mg P450 protein) which were only exposed for three minutes (Figure 2.4). Negative controls included samples with no NADPH and one with no enzymes (Buffer only). These controls showed no activity. Statistical analysis reveled that

there was significant difference (0.0092) between FUMOZ-R and FANG, exposed to 0.75% permethrin (*t*-test, $p < 0.05$). No significant differences (0.1310) were noted between 20 % knockdown FUMOZ-R and FANG (*t*-test, $p > 0.05$) and also PBO-synergized FUMOZ-R and FANG (0.9891) (*t*-test, $p > 0.05$) (Statistix 7, Analytical software version 7.0).

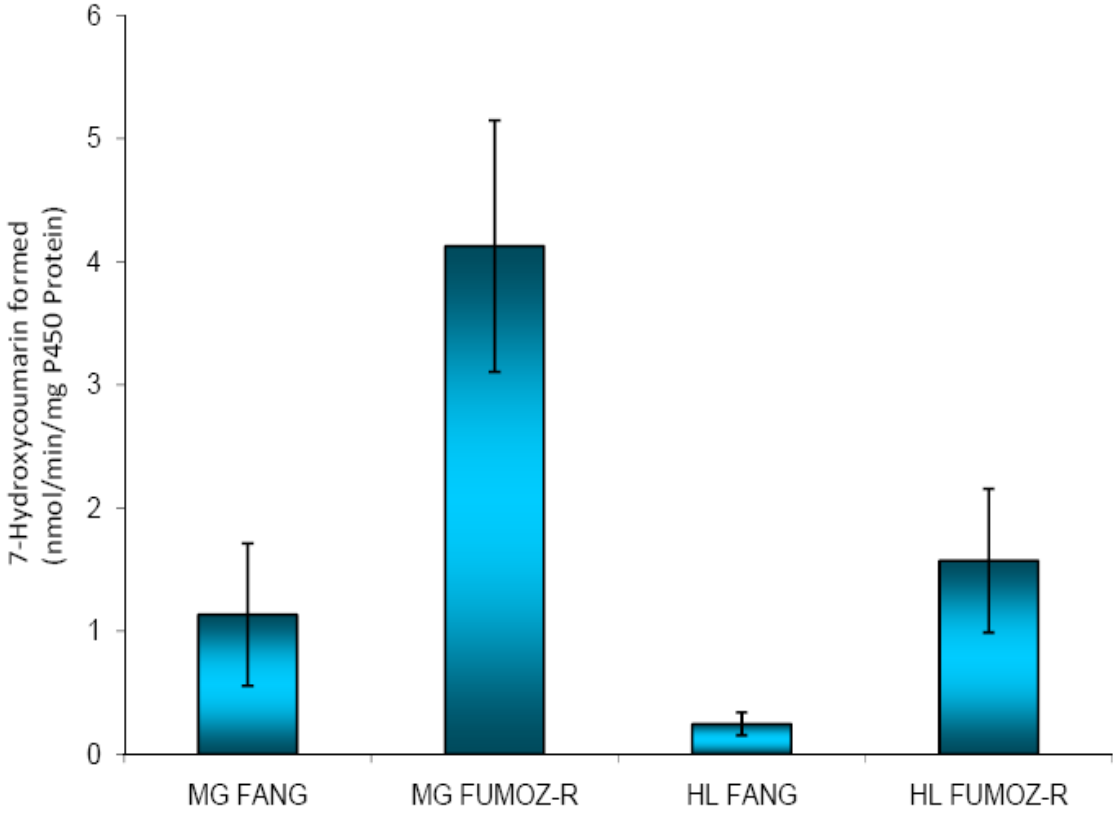


Figure 2.3: P450 assay on total microsomal P450. FUMOZ-R had the highest P450 activity. Larval midgut for both strains had a much higher activity than the adult samples. MG-Midgut and HL-Headless adult.

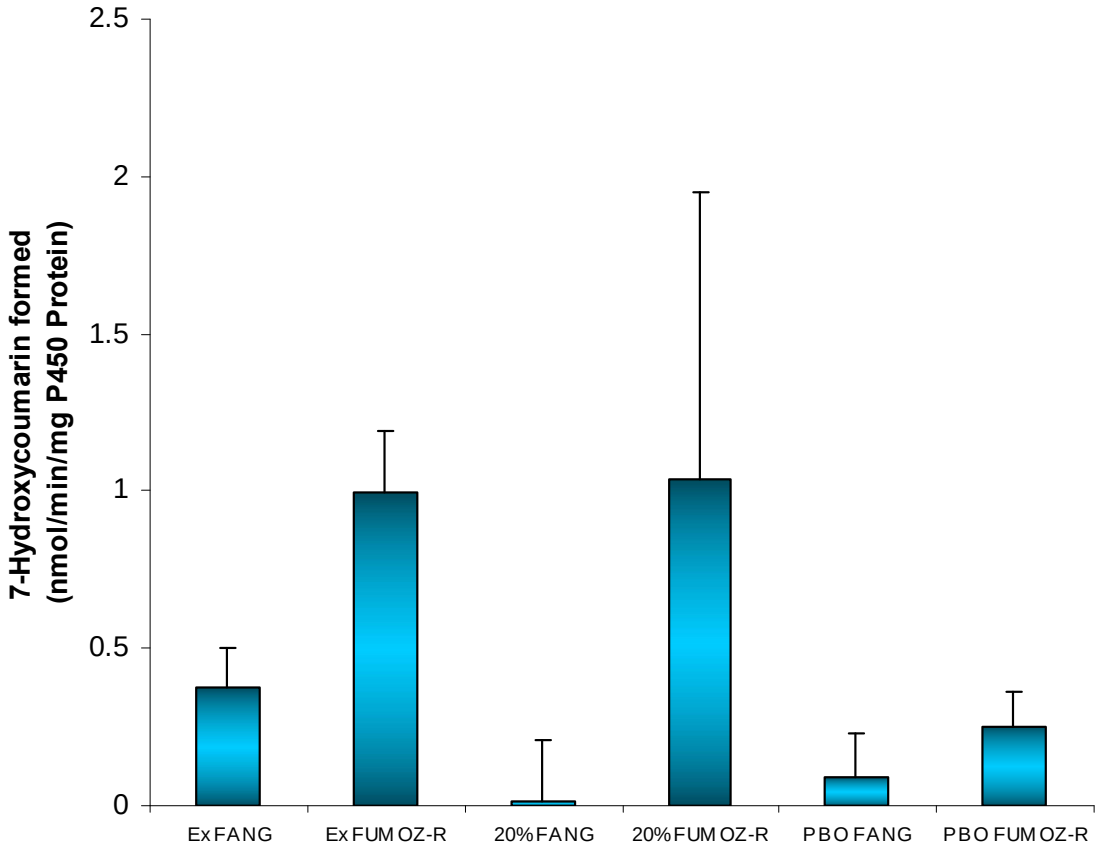


Figure 2.4: P450 assay on mosquitoes exposed to 0.75% permethrin. Mosquitoes were exposed to 0.75% permethrin, 20% knockdown and PBO. FANG had a relatively higher activity with the exposed samples (for 1 hour to permethrin) compared to the 20% knockdown and PBO treated. FUMOZ-R however had the highest activity with the 20% knockdown sample while the exposed sample (1 hour exposed) was slightly lower. FUMOZ-R PBO treated was relatively low compared to the other two. Ex-exposed to 0.75% permethrin for one hour; 20% knockdown – exposed to 0.75% permethrin until 20% mortality was achieved; PBO- exposed to PBO for one hour then exposed to 0.75% permethrin for one hour.

2.4.3 Permethrin metabolism

Permethrin exists as two isomers, *cis* and *trans,* which are detected at about 10.2 and 10.5 mins respectively (Choi *et al.,* 2002; Nakamura *et al.,* 2007). Two permethrin peaks were observed at retention times 10 and 11.3 when loaded on an HPLC column (Figure 2.5A). Headless adult microsomal P450 were used since they were not exposed to permethrin. The microsomal homogenates were incubated with permethrin and analysed after 20 minutes and again 2 ½ hours after incubation. After 2 ½ hours, two small peaks were detected within the FUMOZ-R microsomal P450 at retention times 5.5 and 6 (Figure 2.5B). The peak 1 (6 minutes) formed a shoulder with an existing peak at retention 6.1. This peak (shoulder) was not detected after 30 minute reaction incubation with permethrin. Peak 2 (5.5 minutes) appeared slightly after 30 minutes and increased after 2 ½ hour incubation (Figure 2.5B). Controls containing no microsomal P450 (no enzyme) were included and no peaks were detected at retention times 5.5 and 6. The profile of FANG was similar to that of the control with no peaks.

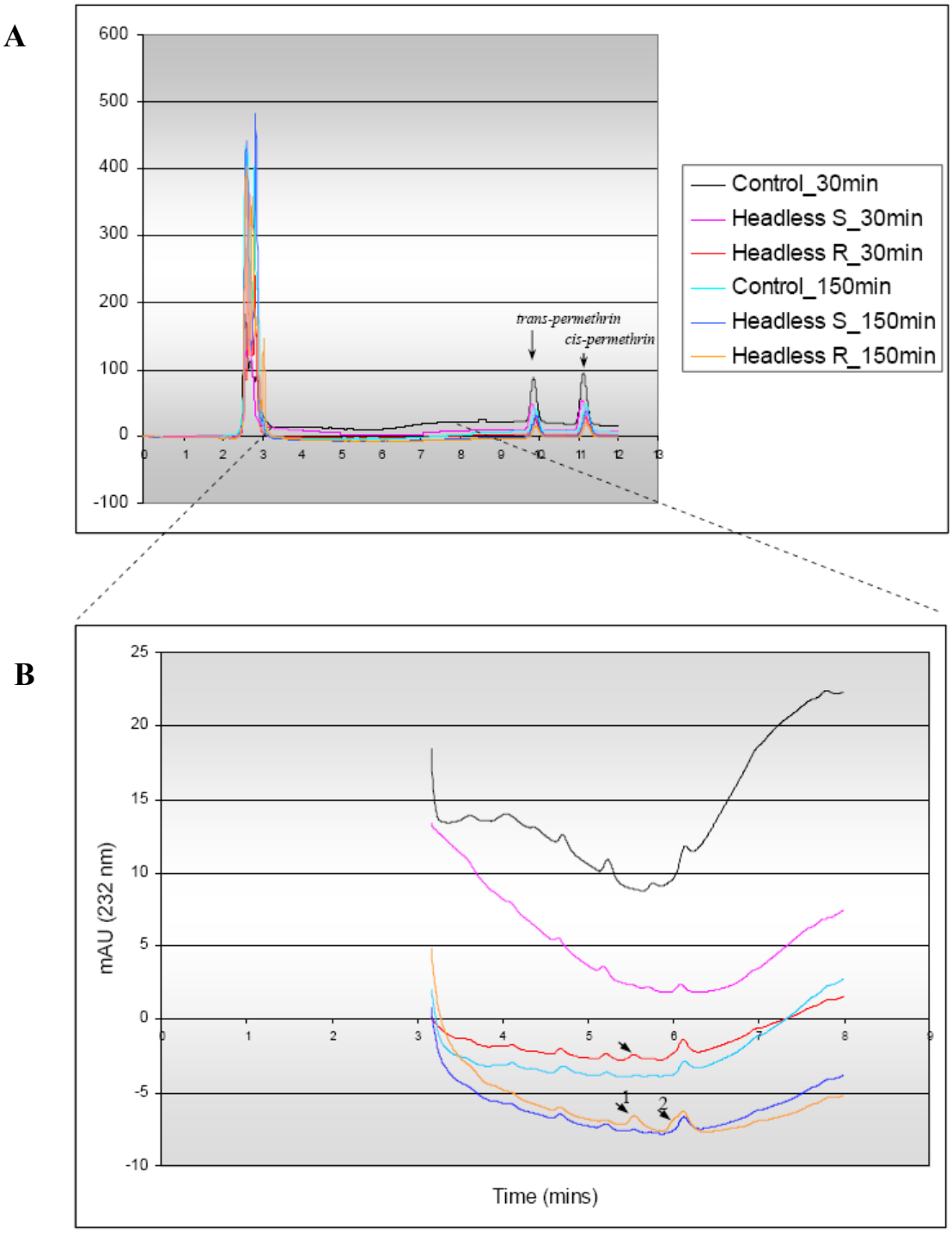


Figure 2.5: HPLC elution profile of permethrin metabolites separated in a C-18 reverse phase column. Mobile phase consisted of acetonitrile/water (90:1). A) Two permethrin peaks are detected at retention times 10 and 11.3. B) The region between retention times 3 and 8 was magnified in order to visualize metabolites produced as a result of the hydrolysis of permethrin. Arrows show peaks formed as a result of permethrin hydrolysis. Metabolites are only present in the FUMOZ-R fraction. S-Susceptible and R-Resistant.

2.4.4 Permethrin metabolism from adult mosquito

Chloroform extraction was used to extract permethrin metabolites from three sets of mosquitoes; i) 0.75% permethrin exposed, ii) PBO treated and iii) 20% knockdown. This was performed on both FUMOZ-R and FANG. However the HPLC used was not sensitive enough to detect distinctive metabolites or permethrin peaks. Presence of metabolites would have been evidence of permethrin metabolism within FUMOZ-R. The peaks from FUMOZ-R (0.75% permethrin and 20% knockdown) could then been compared to determine the effect of each treatment, however this could not be done. A gas chromatography-mass spectrometry (GC-MS), which is sensitive to small amounts of compounds, is required to detect the presence of metabolites from these samples. Other instruments such as an electrospray mass spectrometry (ESMS) and liquid chromatography-electrospray mass spectrometry (LC-ESMS) can also be used. Due to the unavailability of these instruments we were unable to test for permethrin metabolites.

2.5 Discussion

Pyrethroid resistance in *An. funestus* was detected in 1999/2000 (Hargreaves *et al*., 2000). Resistance selection (pyrethroid) was performed by Hunt *et al* (2005) on FUMOZ-R. This colony (FUMOZ-R) as well as the susceptible (FANG) colony was used in this study. Selection pressure has been stopped for eight years and did not seem to decrease resistance over the years. This was noted by a zero percent knockdown after one hour post exposure to 0.75% permethrin and a 100% survival after 24 hours. FANG showed a 100% knockdown after 30 minutes post exposure and 100% mortality after 24 hours confirming its susceptibility status. Increased P450 enzyme activity was shown by Brooke *et al* (2001) to be associated with permethrin resistance in this strain. To confirm that P450s are responsible for pyrethroid resistance in *An. funestus,* a widely used P450 synergist assay using piperonyl butoxide (PBO) was performed (Young *et al.,* 2005). Synergist treated FUMOZ-R showed a characteristic profile similar to that of FANG indicating that P450s were inhibited and were therefore causing the resistant phenotype. Brooke *et al* (2001) also compared PBO synergized with non- synergiszd mosquitoes in *An. funestus* resistant to pyrethroid. They found an increase in mortality with mosquitoes synergized compared to non-synergized.

Microsomes have been isolated from adults and larval midguts from *Culex quinquefasciatus* showing that guts contain higher levels of P450 activity than any other tissues (Kasai *et al*., 2000). This was also noted when analysis of larval midguts from both FUMOZ-R and FANG showed a relatively higher P450 activity

compared to headless adults (4.7 and 2.6 times higher respectively). However this was the opposite for *Drosophila* P450 which had a 20-fold higher activity for adults compared with larvae (Danielson *et al*., 1994). Heads from both adults and larvae were removed due to the eye pigment 'xanthommatin' which is a P450 inhibitor (Danielson and Fogleman, 1994). FUMOZ-R had a much higher activity for both larval midgut and adult with 3.6 and 6.4 fold increases respectively compared with FANG.

The role of P450 in resistance was further supported when high levels of P450 activity were detected in FUMOZ-R in samples exposed to permethrin. A sub-lethal dosage of permethrin (in this study mosquitoes were exposed to 0.75% permethrin until 20% knockdown was obtained) was used to induce P450 genes from both FUMOZ-R and FANG. FUMOZ-R (20% knockdown) was exposed to permethrin for three hours to achieve 20% knockdown. The high enzyme activity may result from P450 induction after exposure, which may also be due to the prolonged permethrin exposure. FANG 20% knockdown was achieved after only three minutes of exposure to permethrin, any longer resulted in an increase in mortality. Exposure to piperonyl butoxide reduced the activity of P450 in both FUMOZ-R and FANG, however low activity was still noted in FUMOZ-R with less than 100% knockdown after 60 minutes. The low activity may be due to the fact that PBO does not inhibit all P450s. This may arise from some mosquitoes not resting on PBO treated papers during exposure.

The general P450 substrate, 7-ethoxycoumarin is known to have different rates of metabolism with different P450s and in some cases no metabolisms at all (Yamazaki *et al*., 1999b). This phenomenon indicates that results obtained from using 7-ethoxycoumain as substrate, to be treated with care. Other substrates such as resorufin also need to be tested alongside 7-ethoxycoumarin**.**

Permethrin metabolism was performed on both FUMOZ-R and FANG total microsomal P450. Permethrin, *trans* and *cis* are detected at times 10.2 and 10.5 minutes respectively on an HPLC (Choi *et al.,* 2002). Two primary metabolites are produced as a result of permethrin metabolism and these are 3-phenoxybenzyl alcohol (retention time 3.7 minutes) and 3-phenoxybenzoic acid (retention time 5.9 minutes). These were however detected in pyrethroid resistant *Nilaparvata lugens* (Vontas *et al.,* 2001). The two metabolite peaks were detected only in FUMOZ-R at 5.5 and 6 minutes. The peak at 6 minutes corresponds to that of 3-phenoxybenzoic acid. FANG however did not produce peaks but had a profile very similar to the negative control (no enzyme) indicating the P450s were not able to metabolize permethrin. No permethrin metabolites were noted from permethrin exposed, PBO treated and 20% knockdown mosquitoes. These experiments were designed to identify metabolites produced as a result of metabolism by P450 enzymes. PBO treated mosquitoes were expected to produce no metabolites. PBO is an inhibitor of P450 hence resulting in no activity. Mosquitoes (20% knockdown) were expected to have induced P450 hence the presence of metabolites within the mosquitoes. Due to the low sensitivity detection of very small amounts of compounds hence permethrin

metabolites were not detected. The results obtained are not conclusive and further anaylsis are required to determine whether FUMOZ-R is capable of metabolizing permethrin.

Metabolite standards 3-phenoxybenzyl alcohol and 3-phenoxybenzoic acid were run on an HPLC but produced messy results with unclear peaks. These results however could not be used to analyze the peaks produced by FUMOZ-R.

Chapter Three

Isolation, identification and characterization of *Anopheles funestus* cytochrome P450s, CYP6P9 and redox partners

3.1 Introduction

3.1.1 Up-regulation of CYP6, P450 genes associated with insecticide resistance

Insect cytochrome P450s have been implicated in the metabolism of insecticides such as DDT and pyrethroids (Scott, 1999). High levels of these enzymes have been reported in insecticide resistant strains compared to those of the susceptible strains (Brooke *et al.,* 2001; Nikou *et al.,* 2003; Rongnoparut *et al*., 2003). Up-regulation of these cytochrome P450s in association with insecticide resistance is not clearly understood (Sabourault *et al*., 2001; Daborn *et al*., 2002).

In *Drosophila melanogaster* CYP6A2 genes in a DDT-resistant strain were expressed at higher levels (20-30 folds) than those of the susceptible strain (Waters *et al*., 1992). Daborn *et al* (2002) also identified CYP6G1 gene to be associated with DDT resistance in *Drosophila*. Two *An. gambiae* strains from the same geological locality were isolated and one was found to be resistant to pyrethroid while the other was susceptible. Resistance to pyrethroid was due to cytochrome P450, CYP6Z1. In the resistant strain CYP6Z1 was expressed 11 and 4.5 times (male and female respectively) higher than the susceptible strain (Nikou *et al*., 2003).

In a multi-resistant *Musca domestica* (Housefly) strain, two P450s associated with pyrethroid resistance (CYP6A1 and CYP12A1) were reported to be constitutively over-expressed at the transcriptional level. This was likely due to a *trans*-regulation event (Sabourault *et al*., 2001). In the same species however, a different P450, CYP6D1 was over-expressed in a pyrethroid resistant housefly (Tomita *et al.,* 1995). This increase in mRNA levels of CYP6D1 in a pyrethroid resistant strain was correlated with an increase in CYP6D1 proteins implicating an increase in transcription.

3.1.2 Mutations associated with resistant cytochrome P450 gene

Although increased levels of cytochrome P450s are generally associated with insect resistance, SNP's (single nucleotide polymorphisms) in these enzyme genes have been associated with resistance. In Drosophila it was shown that a loss in over-production did not necessary mean a loss in resistance. This was demonstrated using CYP6A2 genes that are associated with DDT resistance. In a DDT resistant *Drosophila* strain, CYP6A2 proteins were found expressed 40 times more than the susceptible strain (Bergé *et al*., 1998). Backcrosses (15 selections) performed on resistant ($RDDT^R$) and susceptible *D. melanogaster* produced a strain capable of metabolizing DDT more effectively than susceptible strain. This strain however did not overproduce CYP6A2. Comparing CYP6A2 from resistant and susceptible strains, three amino acid substitutions were noted within the resistant CYP6A2 sequence (Bergé *et al*., 1998). A putative three dimensional model based on bacterial CYP102 known crystal structure was produced. It revealed that the mutations

interfered with the active site. Enzyme activity on wild-type and mutant, CYP6A2 showed an increase in hydroxylation activity with 7-ethoxycoumarin, 7-benzoyloxycoumarin and DDT by mutant CYP6A2 to produce a non-insecticidal dicofol (Bergé *et al*., 1998). Work carried out by Amichot *et al* (2004) revealed that the point mutations (Arg^{335} to Ser^{335}, Leu^{336} to Val^{336} and Val^{476} to Leu^{476}) observed in CYP6A2 were important in the metabolism of DDT. These mutations were clustered within the distal side of helix I close to the substrate recognition cleft.

3.2 Objective

Monooxygenases are one of the major mechanisms involved in insecticide resistance, specifically resistance to pyrethroids. Despite this, very little is known about this complex resistance mechanism. The broad objectives of this study was to isolate, identify and compare conserved amino acids of *An. funestus,* CYP6P9 with those of other insect cytochrome P450s involved in insecticide metabolism using bioinformatics tools such as the Basic Local Alignment Search Tool (BLAST) and primary sequence alignments to identify structural features of potential functional importance. CYP6P9 from resistant and susceptible strains were compared. *Anopheles funestus* redox partners, cytochrome b_5 and NADPH-cytochrome P450 reductase were also isolated and characterized. The redox partners are important because together with P450 enzymes they form a functional P450 system. Putative three-dimensional structures were generated to allow for the identification of domains and predicted orientation of important residues. These genes were also used for later recombinant studies in Chapter 4.

3.3 Materials and Methods

3.3.1 Genomic DNA isolation

Total genomic DNA was extracted from mosquitoes in order to amplify CYP6P9 genomic DNA to determine sequence as well as introns present. In a 1.5 ml microcentrifuge tube, mosquitoes (n = 3) were ground in liquid nitrogen and resuspended in 200 µl extraction buffer (0.08 mM NaCl, 5.47% sucrose, 0.06 M EDTA, 0.5% SDS and 0.1 M Tris-Cl, pH 8.6). The DNA extract was incubated in a water bath (70 ºC) for 30 minutes followed by the addition of potassium acetate (0.01 M) and incubated on ice for 30 minutes. Supernatant was collected by centrifuging at 16,000 *x g* for 10 minutes and 100% ethanol (400 µl) was added. The supernatant was further incubated at -20 ºC for 1 hour followed by centrifuging at 16,000 *x g* for 30 minutes. The pellet was washed in 75% ethanol (200 µl) by centrifuging at 16,000 *x g* for 1 minute then air-dry for 5 minutes. The DNA pellet was then resuspended in TE Buffer (30 µL; 1 mM Tris-Cl and 1mM EDTA, pH 8.0). DNA concentration was quantified using a nanodrop and stored at –20 ºC until ready for PCR amplification (Collins *et al*., 1987).

3.3.2 RNA isolation

RNA was extracted in order to prepare cDNA for the amplification of CYP6P9, cytochrome b_5 and NADPH-cytochrome P450 reductase genes. Total RNA was extracted from three mosquitoes per 200 µl of Tri reagent (Sigma, U.S.A, cat no: T9424). Homogenate was allowed to stand for 5 minutes before adding chloroform

(40 µl), shaking and standing for a further 5 minutes. The homogenate was then centrifuged at 16,000 *x g* for 15 minutes at 4 ºC. The top clear aqueous phase containing RNA was transferred into a clean 1.5 ml microcentrifuge tube and isopropanol (100 µl) was added. The sample was allowed to stand for 5 minutes at room temperature before centrifuging at 16,000 *x g* for 10 minutes at 4 ºC. Supernatant was removed and the RNA pellet was washed in 70% ethanol (200 µl) by centrifuging at 16,000 *x g* for 1 minute. The pellet was then air-dried for 5 minutes and resuspended in 81µl deionized water. RNA was then DNase treated by adding 10x DNase I buffer (8 µl), followed by DNase I enzyme (2 µL) (Sigma, U.S.A, cat no: D5307). The reaction was mixed by vortexing and incubating at 37 ºC for 30 minutes. DNase I was heat killed at 70 ºC for 15 minutes. The DNase treated RNA was cleaned by using RNeasy MiniElute Cleanup kit (QIAGEN, Germany, cat no: 74204). All buffers used in the procedure were supplied as part of the clean up kit. RLT buffer (350 µl) and 100% ethanol (250 µl) were added to the RNA. The RNA was loaded onto an RNeasy column and centrifuged at 16,000 *x g* for 1 minute. The column was then transferred into a new 2 ml collection vial, RPE buffer (750 µl) (content not supplied) was added and centrifuged at 16,000 *x g* for 1 minute. The flow through was discarded and RPE buffer (750 µl) was again added and centrifuged at 16,000 *x g* for 1 minute. The flow through was discarded and the column was centrifuged at 16,000 *x g* for 1 minute. The column was then transferred to a clean 1.5 ml microcentrifuge tube and RNase-free water (50 µl) added and allowed to stand for 1 minute at room temperature. The column was centrifuged at 16,000 *x g* for 1

minute and RNA was collected. RNA concentration was measured using a nanodrop and stored at -80 ºC until ready for use.

3.3.3 Preparation of cDNA for the amplification of open reading frames CYP6P9, cytb_5 and CPR

Synthesis of cDNA was prepared in order to obtain open reading frame of CYP6P9, cytb_5 and CPR. Total RNA was used for synthesizing cDNA. Oligo (dT) adaptor [5' GACTCGAGTCAT CGATTTTTTTTTTTTTTTVN 3'] and dNTP (10 mM) (Inqaba Biotechnical Industries, South Africa) was added to total RNA (2 µL) and incubated at 65 ºC for 5 minutes followed by chilling on ice for 1 minute. First strand buffer (5X; 8 µL), DTT (0.1 M; 2 µL) and RNAsin (0.5 µL) (All supplied by superscript kit, Invitrogen, U.S.A, cat no: 18080-093) were added to the RNA mixture then incubated at 50 ºC for 2 minutes. *Superscript* III RNase H ¯ reverse transcriptase (0.5 µL)(Invitrogen, U.S.A, cat no: 18080-093) was added to the RNA mixture which was then incubated at 55 ºC for 60 minutes and heated to 70 ºC for 15 minutes. Synthesized cDNA (1 µL) was used for the amplification of CYP6P9, Cytochrome b_5 and NADPH-cytochrome P450 reductase genes. Gene specific primers were designed based on the *An. gambiae* sequence to amplify genes of interest (Table 3.1). The same PCR conditions were used for all genes (Table 3.2 and 3.3).

Table 3.1: Primers used for gene amplification

Primer	Sequence	Tm
CYP6P9 Forward	5' ATG GAG CTA ATT AAC GCG GT 3'	58 ºC
CYP6P9 Reverse	5' CTA CAA CTT TTC CAC CTT CA 3'	56 ºC
cytb_5 Forward	5'ATG TCG GGA AGT GAA AAC GTA 3'	56 ºC
cytb_5 Reverse	5' TTA CTG GGT GAA GTA GA ACC 3'	58 ºC
CPR Forward	5' ATG GAC GCC CAG ACA GAA ACG 3'	66 ºC
CPR Reverse	5' TTA GCT CCA CAC GTC CGC CGA GTA T 3'	69 ºC

Table 3.2: PCR protocol (TakaRa Taq ™, Takara Bio Inc, Japan, cat no: R001AM)

Reagents	Volume (µl)	Final concentration
10 x Buffer	5	1x
dNTP mix (10 mM)	5	1 mM
$MgCl_2$ (25 mM)	3	1.5 mM
Forward Primer (3.1 µM)	4	0.248 µM
Reverse Primer (3.1 µM)	4	0.248 µM
Water	variable	
Template DNA	variable	50pg-1µg/50µl
Taq	0.4	0.4 u/µl
Total volume	**50**	

Table 3.3: PCR conditions

Stage	Cycles	Temperature	Time
1	1	94 ºC	2 minutes
2	30	94 ºC	30 seconds
		55.1 ºC	1 minute
		72 ºC	2.5 minutes
3	1	72 ºC	10 minutes

3.3.4 Cloning of CYP6P9, cytb_5 and CPR into pGEM-T Easy Vector

i) Ligation:

Ligation of gDNA and cDNA into pGEM-T Easy vector was performed in order to allow for sequencing as well as subcloning of cDNAs into suitable expression vectors. Amplified PCR products of CYP6P9, cytochrome b_5 and NADPH-cytochrome P450 reductase were separately ligated into pGEM-T Easy Vector (Promega, U.S.A, cat no: A1360). A detail on ligation reaction is provided in Table 3.4.

Table 3.4: pGEM-T Easy vector ligation protocol

Reagents	volume (µl)
2x Rapid Ligation Buffer, T4 DNA Ligase	5
pGEM-T Easy Vector (50 ng)	1
PCR Product (200 ng)	3
T4 DNA Ligase (3 weiss unit/unit)	1
Total volume	10

The reaction was mixed by pipetting and incubated overnight at 4 ºC.

ii) Transformation:

High efficiency competent cells JM 109 (50 µl) were added to ligation reaction (5 µl) in a sterile 1.5 ml tube on ice. The reaction was mixed by gently flicking the tube and storing on ice for 20 minutes. The cells were heat-shocked at exactly 42 ºC in a water bath for 50 minutes and immediately stored on ice for 2 minutes. Luria Bertani (LB) (see Appendix A1 for recipe) broth (950 µl) was added to cells and incubated at 37 ºC for 2 hours with shaking at 200 *rpm*. Transformed cells (100 µl) were plated onto LB plates (see Appendix A1 for recipe) containing ampicillin (AMP) (100 µg/ml),

isopropyl-1-thio-β-D-galactopyranoside (IPTG) (0.5 mM) and 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) (80 μg/ml). The plates were incubated at 37 ºC overnight. Positive clones were selected on the bases of blue and white colonies, with the white colonies containing the insert of interest.

3.3.5 Small scale plasmid isolation of CYP6P9, cytb_5 and CPR

Single white colonies were selected and grown in LB Broth (5 ml) containing AMP at 37 ºC overnight with shaking (200 *rpm*). Plasmids were isolated using the QIAGEN Plasmid Mini kit (QIAGEN, German, cat no: 2125). All buffers used in this procedure were included in this kit. Overnight culture (1 ml) was added to a 1.5 ml microcentrifuge tube and centrifuged at 16,000 *x g* for 1 minute. The supernatant was discarded and pellet was resuspened in 0.3 ml of Buffer P1 (50 mM Tris.Cl, pH8.0; 10 mM EDTA; 100 μg/ml RNase A). Buffer P2 (0.3 ml; 200 mM NaOH, 1% SDS) was added to cells, gently mixed and incubated at room temperature for five minutes in order to lyses cells. Chilled Buffer P3 (0.3 ml; 3.0 mM potassium acetate, pH 5.5) was added, gently mixed and incubated on ice for 5 minutes to allow for cell debris precipitation and renature of circular DNA. Cells were then centrifuged at 16,000 *x g* for 10 minutes. QIAGEN-tip 20 columns were equilibrated by passing through 1 ml of Buffer QBT (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol; 0.15% Triton® X-100) by gravity flow. Supernatant containing plasmids were carefully transferred to equilibrated QIAGEN-tip 20 columns and allowed to enter the resin by gravity flow. DNA bound to the column was washed with 4 ml of Buffer QC (1.0 M

NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol). The columns were then transferred to sterile 1.5 ml tubes, DNA was eluted with 0.8 ml of Buffer QF (1.25 M NaCl; 50 mM Tris.Cl, pH 8.5; 15% isopropanol). Isopropanol (0.56 ml) was added to precipitate DNA, followed by centrifugation at 16,000 *x g* for 30 minutes. DNA pellet was washed with 70% ethanol (1 ml) by centrifuging at 16,000 *x g* for 1 minute then air-dried for five minutes. The pellet was resuspended in ultra pure water (30 µl). Plasmid concentration was quantified using the nanodrop and stored at -20 ºC till ready for use. Glycerol (30%) was added to plasmids and stored at -70C as backup.

3.3.6 Isolation and identification of open reading frame cDNA sequences of CYP6P9, cytb_5 and CPR

Isolated plasmids (pGEM-T/CYP6P9, pGEM-T/cytb_5 and pGEM-T/CPR) were sequenced by Inqaba Biotechnical Industries (South Africa) using pGEM-T Easy vector primers T7 and SP6 (Table 3.5). Once the sequences were known, internal primers for CYP6P9 and CPR were designed using inner regions to sequence the entire internal regions of CYP6P9 and CPR (Table 3.5).

Table 3.5 Primers used to sequence genes

Primer	Sequence	Tm
T7	5' TAA TAC GAC TCA CTA TAG GG 3'	48 ºC
SP6	5' TAT TTA GGT GAC ACT ATA G 3'	50 ºC
CYP6P9 internal Forward	5' CTG TGC ATT CGG GAT TGA G 3'	60 ºC
CYP6P9 internal Reverse	5' CCC TTG CCA ACA CTC CC 3'	62 ºC
CPR internal Forward	5' AAA GTT TTG GCC AAC GGG 3'	57 ºC
CPR internal Reverse	5' GCA GAC GCG GTA ACA GTT 3'	59 ºC

Sequence identity was confirmed by performing a nucleotide-nucleotide BLAST search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Genomic DNA was also sequenced in order to determine number of introns as well as locations.

3.3.7 Bioinformatics studies

Sequenced genes were submitted to Genbank database (http://www.ncbi.nlm.nih.gov/ Genbank/submit.html) to obtain accession numbers. Multiple sequence alignments were performed between protein sequences of interest and those of other organisms that were obtained from the protein database (http://www.ncbi.nlm.nih.gov/ entrez/query) using ClustalW (http://www.ebi.ac. uk/clustalw/). Homology models were generated using SwissModel First Approach Mode (http://swissmodel.expasy. org/SM_FIRST.html) containing known crystal structures as templates that were obtained from the protein data bank (http//www.expasy.org/ swissmod/SM_ Blast.html). PyMOL programme (DeLano, 2002) was used to visualise the generated models.

3.4 Results

3.4.1 Identification of CYP6P9 in *Anopheles funestus,* FUMOZ-R

Amplification of *An. funestus* was achieved using newly designed forward and reverse primers based on *An. gambiae* sequence information on CYP6P3. A fragment of ~1,500bp was amplified using *An. funestus* cDNA template (Figure 3.1). The fragment was cloned into pGEM-T Easy vector and sequenced to verify whether it was a P450 gene. BLAST analysis confirmed that the fragment was 80% identical to that of *An. gambiae* CYP6P3 gene.

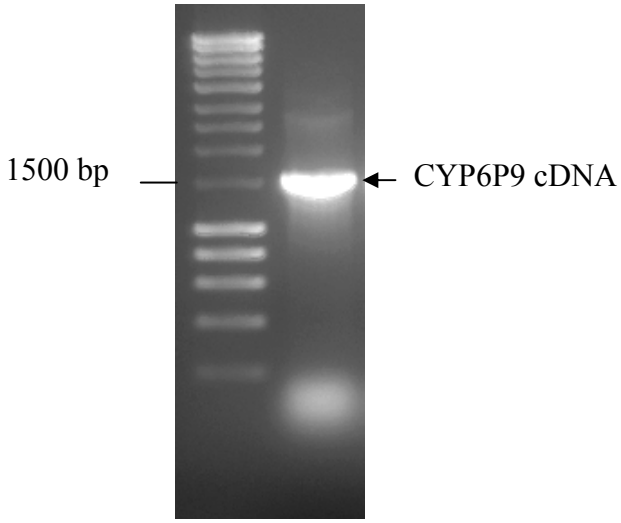


Figure 3.1: Amplified fragment of CYP6P9 cDNA. A fragment size of 1,530 bp was amplified from *An. funestus* cDNA. PCR fragments were run on a 1% TAE agarose gel and viewed under UV light.

Sequence analysis showed that CYP6P9 was 1,530 bp in size. The start (ATG) and stop (TAG) codons were identified using GeneRunner sequence analysis programme (http:// www. generunner.com) which produced three different reading frames. The correct reading frame was identified by start codon ATG which encodes for

methionine (Met) amino acid. The stop codon TAG which does not encode for any amino acid was identified as the end of the open reading frame. The open reading frame of 1,530 bp encoded for a protein of 509 amino acids in length. Start and stop codons used in *An. funestus* P450 genes are shown in Table 3.6.

Table 3.6: Start and stop codons used in *An. funestus* P450 genes

	Codon	Amino acid
Start	ATG	Met
Stop	TGA	End
	TAG	End
	TAA	End

Generatd using http://bioinformatics.org/sms2/codon_usage.html

In order to determine the differences in nucleotide sequence at mRNA level between the resistant (FUMOZ-R) and (FANG) susceptible strains, CYP6P9 cDNA from FANG was also isolated. Both CYP6P9s were characterized as shown in the next section.

3.4.2 Sequence comparison between resistant vs susceptible *Anopheles funestus* CYP6P9

Sequence comparison between CYP6P9 cDNA from FUMOZ-R and FANG was performed in order to identify any nucleotide differences that may affect the amino acid sequence. Only eleven nucleotide differences were noted between the two sequences (Figure 3.2). These differences resulted in four amino acid sequence differences which in turn may result in differences that may be associated with substrate binding or tertiary structure (Figure 3.3). Identification of helices (A-L) and

substrate recognition sites (SRS 1-6) on the secondary structure were determined by sequence aligning CYP6P9 with known crystallographic CYP101A1 sequence as template (Poulos *et al*. 1987; Gotoh, 1992). Only four amino acid variations were found. The first amino acid variation (position 14, Glu to Val) was found near the NH_2 terminus. The second amino acid variation (position 77, Val to Met) was noted between helices A and B. The third variation (position 221, Ile to Phe) was found within the F-G loop near SRS-2. The fourth variation (position 361, Ile to Met) was found within helix K.

An. funestus CYP6P9 genomic DNA (FUMOZ-R) was also amplified and sequenced to determine number of introns as well as sizes. One intron was identified which was 56 bp when aligned with CYP6P9 cDNA sequence. This intron was situated in helix K just before the ETLR domain (Figure 3.3).

```
FUMOZ-R         ATGGAGCTAATTAACGCGGTGTTGGCCGCGTTCATCTTCGTAGTGTCGGCAGTGTACCTT 60
FANG            ATGGAGCTAATTAACGCGGTGTTGGCCGCGTTCATCTTCGAAGTGTCGGCAGTGTACCTT 60
                ************************************ ******************

FUMOZ-R         TTCATTCGGAACAAACATAATTACTGGAAAGACAATGGATTCCCGTATGCGCCGAACCCA 120
FANG            TTCATTCGGAACAAACATAATTACTGGAAAGACGATGGATTCCCGTATGCGCCGAACCCA 120
                ******************************** ************************

FUMOZ-R         CATTTTCTGTTCGGACACGCGAAAGGACAGGCCCAGACAAGGCATGCGGCCGACATCCAT 180
FANG            CATTTTCTGTTCGGACACGCGAAAGGACAGGCCCAGACAAGGCATGCGGCCGACATCCAT 180
                ************************************************************

FUMOZ-R         CTGGAACTGTACAAAAAATTCAAGCAGCGCCGTGAGCGGTACGTTGGTATGAGCCAGTTC 240
FANG            CTGGAACTGTACAAAAAATTCAAGCAGCGCCGTGAGCGGTACGTTGGTGTGAGCCAGTTC 240
                ********************************************** ***********

FUMOZ-R         ATGATACCTTCATTGCTCGTGATCGATCCAGAGCTGGTGAAAACGATCCTAGTAAAGGAC 300
FANG            ATGATACCTTCATTGCTCGTGATCGATCCAGAGCTGGTGAAAACGATCCTAGTAAAGGAC 300
                ************************************************************

FUMOZ-R         TTTAATGTATTCCACGATCATGGTGTATTCAATAATGCAAGAGACGACCCGCTGTCCGCA 360
FANG            TTTAATGTATTCCACGATCATGGTGTATTCAATAATGCAAGAGACGACCCGCTGTCCGCA 360
                ************************************************************

FUMOZ-R         CATCTTTTTGCGCTTGAAGGTAACCCATGGCGCTTGTTGCGTCAGAAGCTCACGCCAACG 420
FANG            CATCTTTTTGCGCTTGAAGGTAACCCATGGCGCTTGTTGCGTCAGAAGCTCACGCCAACG 420
                ************************************************************

FUMOZ-R         TTCACCTCAGGTCGCATGAAGCAAATGTTTGGTACACTATGGGATGTAGCACTTGAGCTG 480
FANG            TTCACCTCAGGTCGCATGAAGCAAATGTTTGGTACACTATGGGATGTAGCACTTGAGCTG 480
                ************************************************************

FUMOZ-R         GACAAGTATATGGAAGAAAACTATCGTCAGCCGGATATTGAGATGAAGGATGTGCTAGGT 540
FANG            GACAAGTATATGGAAGAAAACTATCGTCAGCCGGATATTGAGATGAAGGATGTGCTAGGT 540
                ************************************************************

FUMOZ-R         CGGTTTACGACAGATGTGATTGGCACCTGTGCATTCGGGATTGAGTGTAATACGCTTAAG 600
FANG            CGGTTTACGACAGATGTGATTGGCACCTGTGCATTCGGGATTGAGTGTAATACGCTTAAG 600
                ************************************************************

FUMOZ-R         ACACCGGACTCGGAGTTCCGCAAATACGGCAACAAAGCGTTTGAGTTTAATCTGATGATT 660
FANG            ACACCGGACTCGGAATTCCGCAAATACGGCAACAAAGCGTTTGAGTTTAATCTGATGATT 660
                ************** ***********************************************

FUMOZ-R         TTTCTAAAAACTTTCTTAGCATCGGCTTATCCGTCACTTGTGCGGAAACTGCGAATGAAG 720
FANG            ATTCTAAAAACTTTCTTAGCATCGGCTTATCCGTCACTTGTGCGGAAACCGCGAATGAAG 720
                 ************************************************ **********

FUMOZ-R         ATAACATTCGACGATGTGGAACAGTTTTTCCTAAAAATTGTTAAGGAAACGGTGGAATAT 780
FANG            ATAACATTCGACGATGTGGAACAGTTTTTCCTAAAAATTGTTAAGGAAACGGTAGAATAT 780
                ****************************************************** ******

FUMOZ-R         CGAGAAAGTAACAACATTAAACGAAACGACTTCATGAACCTGCTGTTGCAGATTAAGAAT 840
FANG            CGAGAAAGTAACAACATTAAACGAAACGACTTCATGAACCTGCTGTTGCAGATTAAGAAT 840
                ************************************************************

FUMOZ-R         AAGGGTAAGCTGGACGACAGCGATGATGGGAGTGTTGGCAAGGGTGAAGTAGGAATGACA 900
FANG            AAGGGTAAGCTGGACGACAGCGATGATGGGAGTGTTGGCAAGGGTGAAGTAGGAATGACA 900
                ************************************************************

FUMOZ-R         CAACGAGAACTTGCGGCACAGGCATTCATTTTCTTCTTGGCCGGTTTCGAGACATCATCC 960
FANG            CAACGAGAACTAGCGGCACAGGCATTCATTTTCTTCTTGGCCGGTTTCGAGACATCATCC 960
                *********** ************************************************

FUMOZ-R         ACGACGCAAAGCTTCTGTCTGTACGAGTTGGCAAAGAACCCTGACATCCAGGAGCGCCTT 1020
FANG            ACGACGCAAAGCTTCTGTCTGTACGAGTTGGCAAAGAACCCTGACATCCAGGAGCGCCTT 1020
                ************************************************************
```

```
FUMOZ-R         AGACAAGAGATCAACCAAGCAATCGAGGAGAATGACGGCCAGGTGACGTACGATGTCGCC 1080
FANG            AGACAAGAGATCAACCAAGCAATCGAGGAGAATGACGGCCAGGTGACGTACGATGTCGCC 1080
                ************************************************************

FUMOZ-R         ATGAACATACAGTATCTGGACAATGTGATAAACGAAACACTTCGCAAGTACCCACCGGTA 1140
FANG            ATAAACATACAGTATCTGGACAATGTGATAAACGAAACACTTCGCAAGTACCCACCGGTA 1140
                ** ************************************************************

FUMOZ-R         GAATCGTTGAGTCGTGTGCCGTCTGTTGACTATGTCATCCCAGGTACGAAACATGTCATT 1200
FANG            GAATCGTTGAGTCGTGTGCCGTCTGTTGACTATGTCATCCCAGGTACGAAACATGTCATT 1200
                ************************************************************

FUMOZ-R         CCCAAACGAACGTTAGTGCAAATTCCGGTTCACGCCATTCAACATGATCCTGAGCACTAT 1260
FANG            CCCAAACGAACGTTAGTGCAAATTCCGGTTCACGCCATTCAACATGATCCTGAGCACTGT 1260
                ************************************************************ *

FUMOZ-R         CCCGATCCAGAACGTTTCGATCCGGATCGCTTCTCACCGGAGGAAGTGAAGAAGCGACAT 1320
FANG            CCCGATCCAGAACGTTTCGACCCGGATCGCTTCTCACCGGAGGAAGTGAAGAAGCGACAT 1320
                ******************* ************************************

FUMOZ-R         CCCTTCACGTTCCTCCCATTCGGTGAGGGGCCACGCGTTTGCATTGGGCTTCGGTTTGGT 1380
FANG            CCCTTCACGTTCCTCCCATTCGGTGAGGGGCCACGCGTTTGCATTGGGCTTCGGTTTGGT 1380
                ************************************************************

FUMOZ-R         GTGATGCAGACGAAGGTAGGATTGATAACGCTGTTGAGAAAGTTCCGCTTCTCACCGTCA 1440
FANG            GTGATGCAGACGAAGGTAGGATTGATAACGCTGTTGAGAAAGTTCCGCTTCTCACCGTCA 1440
                ************************************************************

FUMOZ-R         GCGCGTACACCAGATTGTGTAAAGTTCGATCCGAAAATGATCATTCTGTCACCGATCGCG 1500
FANG            GCGCGTACACCAGATTGTGTAAAGTTCGATCCGAAAATGATCATTCTGTCACCGATCGCG 1500
                ************************************************************

FUMOZ-R         GGTAATTACTTGAAGGTGGAAAAGTTGTAG 1530
FANG            GGTAATTACTTGAAGGTGGAAAAGTTGTAG 1530
                ******************************
```

Figure 3.2: Nucleotide comparison of CYP6P9 cDNA from resistant and susceptible strains. Differences are noted in grey shade and identical nucleotides are represented with asterisk. FUMOZ-R (GeneBank accession number: AY729661) and FANG (GeneBank accession number: EU450763) (ClustalW,http://www.ebi.ac.uk/clustalw/).

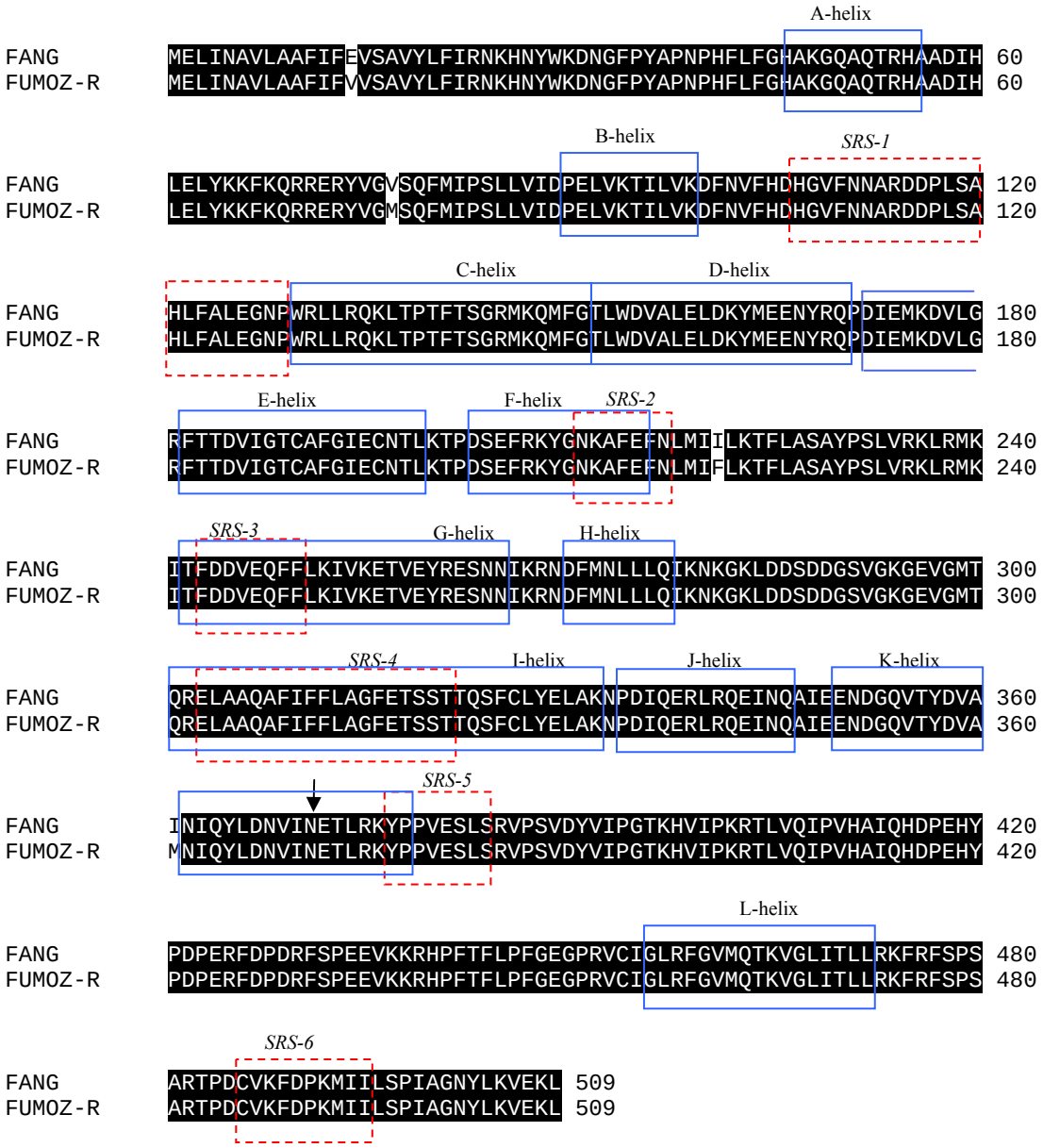


Figure 3.3: Amino acid comparison of CYP6P9 from resistant and susceptible strains. Helices A-L are represented by ( ) and substrate recognition sites (SRS) 1-6 are indicated by ( ). Position of intron is indicated by (↓). Identical residues are shaded in black while different residues are in white (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html).

3.4.3 Sequence characterization of *Anopheles funestus* CYP6P9

When aligned with *An. gambiae* CYP6P3 (the ortholog to CYP6P9) (Ameya *et al.,* 2005), *An. funestus* CYP6P9s shared an 84.9% identity. When CYP6P9 (FUMOZ-R) was compared to other insect CYP6 proteins involved in insecticide resistance it revealed identities of: 45.5%, 34.0%, 33.5% and 40.8% with CYP6A1, *Musca domestica* (Feyereisen *et al*., 1989); CYP6Z1, *Anopheles gambiae* (Ranson *et al*., 2002); CYP6D1, *Musca domestica* (Tomita *et al*., 1995) and CYP6F1 *Culex pipiens pallens* (Gong *et al*., 2005) respectively (Table 3.7). This is expected since these P450 systems are all from different subfamilies.

Table 3.7: Percentage homology amongst various insect CYP6 proteins

	CYP6D1	CYP6Z1	CYP6F1	CYP6P3	FUMCYP6P9	CYP6A1	FANCYP6P9
CYP6D1	-	41.7	39.3	32.7	33.5	30.0	33.5
CYP6Z1	41.7	-	40.1	33.8	34.0	33.7	34.0
CYP6F1	39.3	40.1	-	39.6	40.8	34.0	40.8
CYP6P3	32.7	33.8	39.6	-	84.9	45.9	84.9
FUMCYP6P9	33.5	34.0	40.8	84.9	-	45.5	99.3
CYP6A1	30.0	33.7	34.0	45.9	45.5	-	45.5
FANCYP6P9	33.5	34.0	40.8	84.9	99.3	45.5	-

Data assembled using LASERGENE package (DNASTAR, MegAlign program, Clustal W method)

Amino acid multiple sequence alignments of CYP6P9 against other insect CYP6s show that they share the conserved regions characteristic of cytochrome P450 proteins (Figure 3.4). These conserved regions are the heme-binding region (PFxxGxxxCxG) (position 446-456) (positions are based on FUMOZ-R/FANG sequences), which contain the highly conserved cysteine residue that holds the heme-

group in place; the ExxR motif (position 372-375) situated within helix K that stabilizes the heme cavity structure, an oxygen binding pocket (proton transfer groove) ((A/G)Gx(E/D) T(T/S)) (position 314-319), on the distal side of the heme group within helix I which is also known as the P450 signature. The YPDP motif, found in the CYP6 family (position 420-423) has an aromatic group (Tyr or Phe) and two proline amino acids that are conserved. A microsomal cytochrome P450 motif (PERF) (position 428-431) was found downstream of the YPDP motif. In the CYP6P9s and CYP6P3, Asp^{429} replaces Glu (Werck-Reichhart and Feyereisen, 2000). The N-terminal region constitutes 61% hydrophobic amino acids and is involved in membrane anchoring (Rongnoparut *et al*., 2003).

Hydrophobic N-Terminal anchor region

```
CYP6D1          -----MLLLLLLIVVTTLYIFAKLHYTKWERLGFESDKATIPLGSMAKVFHKERPFGLVM 55
CYP6Z1          -----MILYTIGLIVTFVFLALKYVYSYWDRQGLPNLRPEIPYGNLRILAQKKESFNVAI 55
CYP6F1          ---MFAWIICAAAAVPLVYFLIVYQFSYWKRRGITQLTPSFPFGDLGPFFRQRSSLGVVY 57
FUMCYP6P9       MELINAVLAAFIFVVSAVYLFIRNKHNYWKDNGFPYAPNPHFLFGHAKGQAQTRHAADIH 60
FANCYP6P9       MELINAVLAAFIFEVSAVYLFIRNKHNYWKDNGFPYAPNPHFLFGHAKGQAQTRHAADIH 60
CYP6P3          MELINAVLAAFIFAVSIVYLFIRNKHNYWKDNGFPYAPNPHFLFGHAKGQAQTRHGADIH 60
CYP6A1          MDFGSFLLYALGVLASLALYFVRWNFGYWKRRGIPHE-EPHLVMGNVKGLRSKYHIGEII 59
                       :      ..         .  *.  *:          .      .

CYP6D1          SDIYDKC--HEK-VVGIYLFFKPALLVRDAELARQILTTDFNSFHDRGLYVDEKNDPMSA 112
CYP6Z1          NDLYDRS--SER-LVGVYLFFRPAILVRDAHLAKRIMVNDFQHFHDRGVYCNEHSDPMSA 112
CYP6F1          ADVYRLC--KRLPFVGIYFSLRPMLVVNDPELIKNVLVRDFDHFHDRGLYVNEEKDPLSG 115
FUMCYP6P9       LELYKKFKQRRERYVGMSQFMIPSLLVIDPELVKTILVKDFNVFHDHGVFNNARDDPLSA 120
FANCYP6P9       LELYKKFKQRRERYVGVSQFMIPSLLVIDPELVKTILVKDFNVFHDHGVFNNARDDPLSA 120
CYP6P3          QELYRYFKQRGERYGGISQFIVPSVLVIDPELAKTILVKDFNVFHDHGVFTNAKDDPLTG 120
CYP6A1          ADYYRKFKGSGP-FAGIFLGHKPAAVVLDKELRKRVLIKDFSNFANRGLYYNEKDDPLTG 118
                 : *           *:     *  :* * .* : ::  **. * ::*:: : ..**::.

CYP6D1          NLFVMEGQSWRTLRMKLAPSFSSGKLKGMFETVDDVADKLINHLNERLKDGQTHVLEIKS 172
CYP6Z1          NLFALPGQRWKNLRAKLTPTFTSGQLRHMLPTFLAVGSKLEQYL-ERLAN-EKQIVDMRD 170
CYP6F1          HLFALGGEQWRHHRSKLTPTFTSGRLKEMFTNLVQIGRVLQDHVAKRAGE----DIEIRD 171
FUMCYP6P9       HLFALEGNPWRLLRQKLTPTFTSGRMKQMFGTLWDVALELDKYMEENYRQP---DIEMKD 177
FANCYP6P9       HLFALEGNPWRLLRQKLTPTFTSGRMKQMFGTLWDVALELDKYMEENYRQP---DIEMKD 177
CYP6P3          HLFALEGQPWRLMRQKLTPTFTSGRMKQMFGTIWDVGIELEKCMEQSYNQP---EVEMKD 177
CYP6A1          HLVMVEGEKWRSLRTKLSPTFTAGKMKYMYNTVLEVGQRLLEVMYEKLEVSS--ELDMRD 176
                :*. : *: *:  * **:*:*::*::: *  ..  :.  * . : :         ::::.

CYP6D1          ILTTYAVDIIGSVIFGLEIDSFTHPDNEFRVLSDRLFNPKKSTMLERIRNLSTFMCPPLA 232
CYP6Z1          IVSRYVLDVVASVFFGFEANCLHDPDDAFRVALRDLNNP--DSFINNIRTAGVFLCPGLL 228
CYP6F1          VMARYTTDIIASVGFGIENDSINEKGNIFREMGTKVFSP---DLKTILRLTSTFFTPKLN 228
FUMCYP6P9       VLGRFTTDVIGTCAFGIECNTLKTPDSEFRKYGNKAFEFN---LMIFLKTFLASAYPSLV 234
FANCYP6P9       VLGRFTTDVIGTCAFGIECNTLKTPDSEFRKYGNKAFEFN---LMIILKTFLASAYPSLV 234
CYP6P3          ILGRFTTDVIGTCAFGIECNTLKTTDSEFRKYGNKAFELN---TMIMMKTFLASSYPTLV 234
CYP6A1          ILARFNTDVIGSVAFGIECNSLRNPHDRFLAMGRKSIEVP---RHNALIMAFIDSFPELS 233
                ::  :  *::.:  **:* : :    . *        .         :        * *

CYP6D1          KLLSRLGAKDPITYRLRDIVKRTIEFREEKGVVRKDLLQLFIQLRNTGKISDDNDKLWHD 292
CYP6Z1          KFTGINSLSPPMKKFTTEVISSHLHQRETGQVTRKDFIQMLTDLR------------RK 275
CYP6F1          ALFGFKFIAQEIEDFIMNVVRETLEYRESNKVVRKDMMQLLMQLRNSGTVSIDDR---WD 285
FUMCYP6P9       RKLRMKITFDDVEQFFLKIVKETVEYRESNNIKRNDFMNLLLQIKNKGKLDDSDD----- 289
FANCYP6P9       RKLRMKITFDDVEQFFLKIVKETVEYRESNNIKRNDFMNLLLQIKNKGKLDDSDD----- 289
CYP6P3          RNLHMKITYNDVERFFLDIVKETVDYREANNVKRNDFMNLMLQIKNKGKLDDSDD----- 289
CYP6A1          RKLGMRVLPEDVHQFFMSSIKETVDYREKNNIRRNDFLDLVLDLKN----NPESI----- 284
                          :     . :   :. **   : *:*::::. :::

CYP6D1          VESTAENLKAMSIDMIASNSFLFYIAGSETTAATTSFTIYELAMYPEILKKAQSEVDECL 352
CYP6Z1          AGSSGE--ETLTDAQCAANVFLFYGAGADTSTGTITFTLHELTHNAEAMAKLQQEVDEMM 333
CYP6F1          IEVSTNK-KKLSLEQVTAHAFVFFIAAYETSSTTISFCLFELARNPEIQKKVQQEIDQVL 344
FUMCYP6P9       -GSVGKGEVGMTQRELAAQAFIFFLAGFETSSTTQSFCLYELAKNPDIQERLRQEINQAI 348
FANCYP6P9       -GSVGKGEVGMTQRELAAQAFIFFLAGFETSSTTQSFCLYELAKNPDIQERLRQEINQAI 348
CYP6P3          -GSLGKGEVGMTQNELAAQAFVFFLAGFETSSTTQSFCLYELAKNPDIQERLREEINRAI 348
CYP6A1          -SKLG----GLTFNELAAQVFVFFLGGFETSSTMGFALYELAQNQQLQDRLREEVNEVF 339
                          ::    ::: *:*: .. :*:: *  * :.**:   :   : :.*::. :

CYP6D1          QRHGLKPQGRLTYEAIQDMKYLDLCVMETTRKYPGLPFLNRKCTQDFQVPD-TKLTIPKE 411
CYP6Z1          ERH----HGEITYDNINGMKYLDLCVKETLRIYPALAVLNRECTIDYKVPD-SDTVIRKG 388
CYP6F1          ASH----NGEITYDNINEMKYLENCIDETLRKYPAVPFLNRECSKDYKIPG-TDTTIEKG 399
FUMCYP6P9       EEN----DGQVTYDVAMNIQYLDNVINETLRKYPPVESLSRVPSVDYVIPG-TKHVIPKR 403
FANCYP6P9       EEN----DGQVTYDVAINIQYLDNVINETLRKYPPVESLSRVPSVDYVIPG-TKHVIPKR 403
CYP6P3          AEN----GGEVTYDVVMNIKYLDNVIDETLRKYPPVESLTRVPSVDYLIPG-TKHVIPKR 403
CYP6A1          DQFK---EDNISYDALMNIPYLDQVLNETLRKYPVGSALTRQTLNDYVVPHNPKYVLPKG 396
                       ..::*:    : **:  : ** * **    *.*    *: :*  .. .: *
```

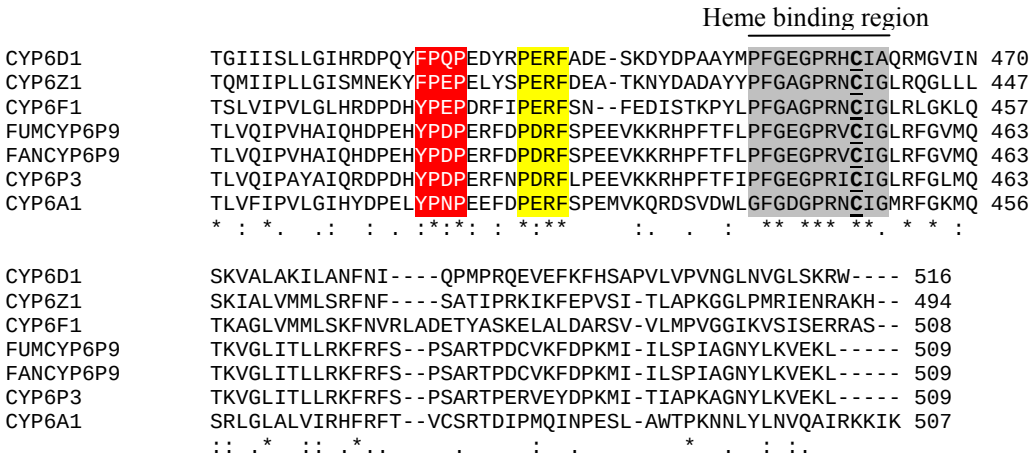


Figure 3.4: Comparison of the open reading frame amino acid sequence of CYP6P9 with other insect CYP6s. The YPDP domain conserved in CYP6 genes only is represented in red, heme-binding site (PFxxGxxxCxG) is indicated in grey while the cysteine involved in heme coordination is highlighted in bold and underlined. The highly conserved ETLR and PERF domains are shaded in black and yellow respectively. The P450 signature AGxETS is represented in blue. The hydrophobic anchor region (N-terminus) is in bold. Identical residues are indicated with asterisks. *Musca domestica*: CYP6D1 (GeneBank accession number: AAC46931), *Anopheles gambiae*: CYP6Z1 (GeneBank accession number: AAL93296), *Culex pipiens pallens*: CYP6F1 (GeneBank accession number: AAX47271), *Anopheles gambiae*: CYP6P3 (GeneBank accession number: AAL93295), *Anopheles funestus* (FUMOZ-R): FUMCYP6P9 (GeneBank accession number: AY729661), *Anopheles funestus* (FANG): FANCYP6P9 (GeneBank accession number: EU450763), *Musca domestica*: CYP6A1 (GeneBank accession number: AAA29293). (ClustalW,http://www.ebi.ac.uk /clustalw/).

3.4.4 Isolation of a CYP6 gene (CYP6P13) from *An. funestus* (FANG) similar to CYP6P9

Forward and reverse primers for CYP6P9 (Table 3.1) were used to isolate CYP6P9 from FANG genomic DNA so as to compare intron sequences as well as size with that of FUMOZ-R gDNA. The amplified gene was sequenced and BLAST analysis identified it to have an 80% identity to that of CYP6P3. Comparison revealed the presence of a second CYP6P9 gene. Analysis between FANG cDNA and FANG gDNA showed numerous nucleotide differences and a 93.7% identity (Figure 3.5). FANG gDNA had one intron which was of 57 bp in length. When FANG gDNA intron was compared with FUMOZ-R gDNA intron, it was one bp longer and had

numerous differences. This led to the suggestion that a novel P450 gene similar to CYP6P9 had been isolated. The novel CYP6 gene was sent for naming to the P450 Nomenclature committee and named CYP6P13.

Sequence alignment was performed at the deduced amino acid level between FANG-CYP6P9 and FANG-CYP6P13. A total of 32 amino acid differences were noted between FANG-CYP6P9 and FANG-CYP6P13 (Figure 3.6). The novel FANG-CYP6P13 had all the characteristics of the CYP6 family. These were noted by the presence of a heme-binding region (PFxxGxxxCxG), ExxR motif that stabilizes the heme cavity structure, an oxygen binding pocket (AGxETS) also known as the P450 signature. The YPDP motif present only in the CYP6 family and microsomal cytochrome P450 motif (PERF). CYP6P13 was sent together with CYP6P9 for naming so as to be classified correctly. The four amino acid differences noted between FANG and FUMOZ-R were compared with CYP6P13. They were found to be identical to those in FUMOZ-R-CYP6P9, except for F^{221} which is substituted with M^{221}.

```
CYP6P9          ATGGAGCTAATTAACGCGGTGTTGGCCGCGTTCATCTTCGAAGTGTCGGCAGTGTACCTT
CYP6P13         ATGGAGCTAATTAACGCGGTGTTGGCCGCGTTCATCTTCGTAGTGTCGGCAGTGTACCTT
                ****************************************:*******************

CYP6P9          TTCATTCGGAACAAACATAATTACTGGAAAGACGATGGATTCCCGTATGCGCCGAACCCA
CYP6P13         TTCATTCGGAACAAACATAATTACTGGAAAGACAATGGATTCCCGTATGCGCCGAATCCG
                **********************************.********************** **.

CYP6P9          CATTTTCTGTTCGGACACGCGAAAGGACAGGCCCAGACAAGGCATGCGGCCGACATCCAT
CYP6P13         CATTTTCTGTTCGGACACGCGAAAGGACAGGCCCAGACAAGGCATGCGGCCGACATCCAT
                ************************************************************

CYP6P9          CTGGAACTGTACAAAAAATTCAAGCAGCGCCGTGAGCGGTACGTTGGTGTGAGCCAGTTC
CYP6P13         CTGGAACTGTACAAAAAATTCAAGCAGCGCGGTGATCGGTACGTTGGTATGAGCCAGTTC
                ***************************** **** ************.***********

CYP6P9          ATGATACCTTCATTGCTCGTGATCGATCCAGAGCTGGTGAAAACGATCCTAGTAAAGGAC
CYP6P13         ATCATACCATCTGTGTTTGTGATCGATCCAGAGCTGGTGAAAACGATCCTAGTAAAGGAC
                ** *****:**: ** * ****************************************

CYP6P9          TTTAATGTATTCCACGATCATGGTGTATTCAATAATGCAAGAGACGACCCGCTGTCCGCA
CYP6P13         TTTAATGTATTCCACGATCGCGGTATTTTCACTAATGCAAGAGACGATCCACTATCAGGA  <-- Forward Primer2
                ******************. ***.*:****.*************** **.**.**.* *

CYP6P9          CATCTTTTTGCGCTTGAAGGTAACCCATGGCGCTTGTTGCGTCAGAAGCTCACGCCAACG
CYP6P13         CACTTGTTTGCGCTGGAAGGTAACCCATGGCGCTTGTTGCGTCAGAAGCTCACGCCAACG
                **  * ******** ********************************************

CYP6P9          TTCACCTCAGGTCGCATGAAGCAAATGTTTGGTACACTATGGGATGTAGCACTTGAGCTG
CYP6P13         TTCACCTCAGGTCGCATGAAGCAAATGTTTGGTACATTATGGGATGTAGCACTTGAGCTG
                ********************************** ***********************

CYP6P9          GACAAGTATATGGAAGAAAACTATCGTCAGCCGGATATTGAGATGAAGGATGTGCTAGGT
CYP6P13         GACAAGTATATGGAAGAAAACTACCATCAGCAGGAGATTGAGATGAAGGATGTGCTAGGT
                ********************** *.*****.*** ************************

CYP6P9          CGGTTTACGACAGATGTGATTGGCACCTGTGCATTCGGGATTGAGTGTAATACGCTTAAG
CYP6P13         CGGTTTACGACCGATGTGATTGGCACGTGCGCATTCGGCATCGAGTGTAATACGCTTAAG
                ***********.************** ** ******** ** ******************

CYP6P9          ACACCGGACTCGGAATTCCGCAAATACGGCAACAAAGCGTTTGAGTTTAATCTGATGATT  <-- Forward Primer1 and Reverse Primer2
CYP6P13         ACACCGGACTCGGAATTCCGCAAATACGGTAACAAAGCGTTCGAGCTGGATCTCCTAATT
                **************************** *********** *** * .**** .*.***

CYP6P9          ATTCTAAAAACTTTCTTAGCATCGGCTTATCCGTCACTTGTGCGGAAACCGCGAATGAAG
CYP6P13         ATGATGAAGTTTTTCTTTGCATCGGCTTATCCGTCACTTGTGCGGAAACTGCGAATGAAG
                ** .*.**.: ******:********************************** **********

CYP6P9          ATAACATTCGACGATGTGGAACAGTTTTTCCTAAAAATTGTTAAGGAAACGGTAGAATAT
CYP6P13         ATCACATTCGATGATGTGGAAGAGTTTTTCGTAAAAATAGTCCGCGAGACGGTGAACTAT  <-- Reverse Primer1
                **.******** ******** ******** *******:** .. **.*****..*.***

CYP6P9          CGAGAAAGTAACAACATTAAACGAAACGACTTCATGAACCTGCTGTTGCAGATTAAGAAT
CYP6P13         CGTGAAATGAACAATGTAAAACGAAACGACTTCATGAACCTGCTGTTGCAGATCAAGAAT
                **:****  *****  .*:********************************** ******

CYP6P9          AAGGGTAAGCTGGACGACAGCGATGATGGGAGTGTTGGCAAGGGTGAAGTAGGAATGACA
CYP6P13         AAGGGCAAGCTGGACGACAGTGATGATGGGAGTGTTGGCAAAGGTGAAGTAGGAATGACA
                ***** ************** ********************.******************
```

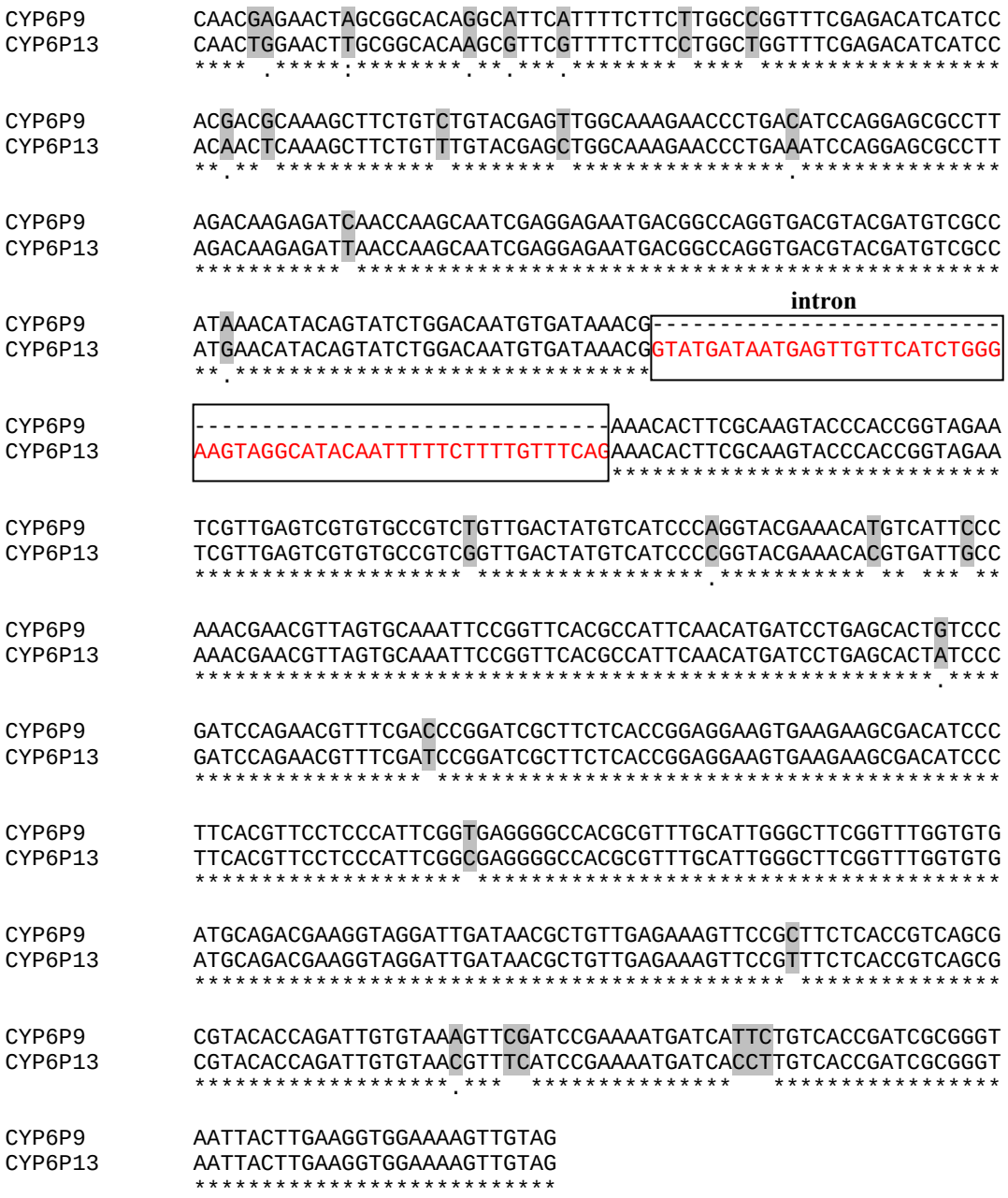


Figure 3.5: Nucleotide sequence alignment of FANG-CYP6P9 and FANG-CYP6P13 genomic DNAs. CYP6P9 cDNA aligned with CYP6P13 gene isolated from FANG. Intron is indicated in red and box. Primers were designed based on nucleotides in blue to differentiate the two genes. Differences are noted in grey shade and identical nucleotides are indicated with asterisks while similar residues are indicated with dot. CYP6P9 (GeneBank accession number: EU450763) and CYP6P13 (GeneBank accession number: EF152577) (ClustalW, http://www.ebi.ac.uk/clustalw/).

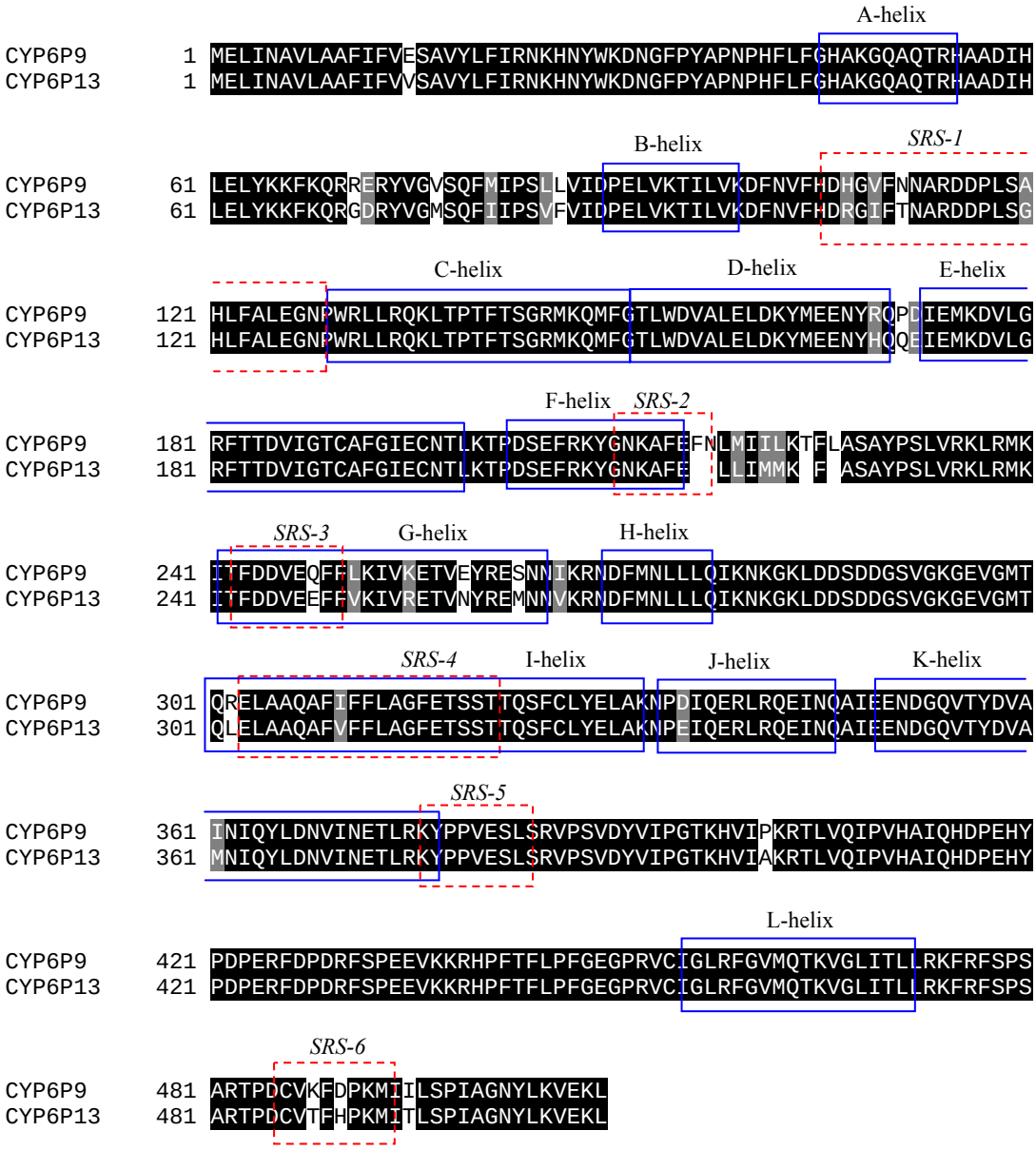


Figure 3.6: Amino acid sequence alignment of FANG-CYP6P9 and FANG-CYP6P13. Helices A-L are represented by ( ) and substrate recognition sites (SRS) 1-6 are indicated by ( ). Identical residues are shaded in black while similar residues are in grey shades. (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html).

With the identification of the novel CYP6P13 gene, it was no longer clear whether the over-expressed CYP6 gene in FUMOZ-R was that of CYP6P9 or CYP6P13. Primers were designed in order to determine whether CYP6P13 was present in both FANG and FUMOZ-R. This was achieved by designing primers based on regions of

high differences (Figure 3.5) (Table 3.8). These primers were used to amplify short segments of about 130 bp and 300 bp. After numerous PCR trials (changing annealing temperatures and $MgCl_2$ concentrations) using FUMOZ-R cDNA and genomic DNA no fragments were amplified. A fragment of 300 bp was amplified using FANG genomic DNA (Figure 3.7). This fragment was sequence and found to be identical to that of FANG-CYP6P13 gene. The 130 bp fragment amplified was sequenced but sequencing failed. However no fragment was amplified from FANG cDNA.

Table 3.8: Primers used to amplify fragments CYP6P13 gene

Primers	Sequence
Forward primer1	5' CTGGATCTCCTAATTATGATGAAGTTTTTC 3'
Reverse primer1	5' GTTCACCGTCTCGCGGACT 3'
Forward primer2	5'ACTATCAGGA CACTTG 3'
Reverse primer2	5' GAAAAACTTCATCATAATTAGGAGATCCAG 3'

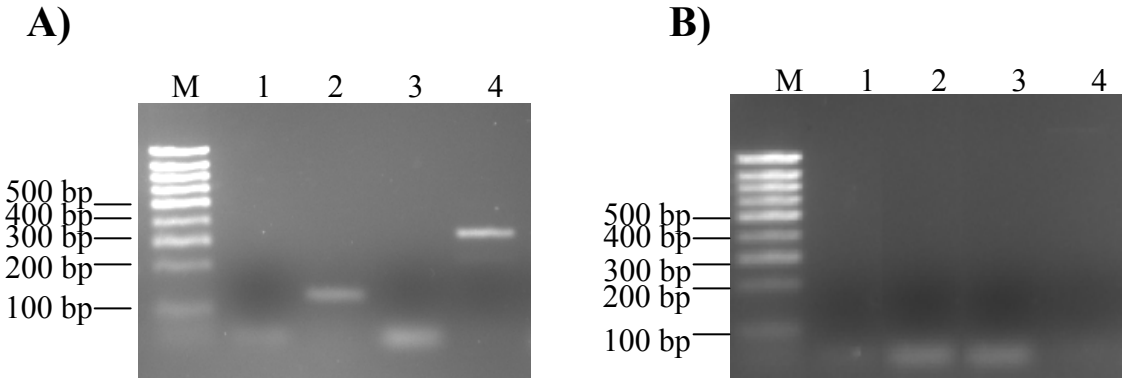


Figure 3.7: Amplification of CYP6P13 fragments from cDNA and gDNA. Two sets of primers were used to amplify a 130 bp and 300 bp fragments from CYP6P13. A) Amplification using FANG cDNA and gDNA. Lane M: Molecular marker, lanes 1 and 3: FANG cDNA, lanes 2 and 4: FANG gDNA. B) FUMOZ-R; Lane M: Molecular marker, lanes 1 and 3: FUMOZ-R cDNA, lanes 2 and 4: FUMOZ-R gDNA. DNA was run on a 1% TAE agarose gel and viewed under UV light.

A phylogenetic tree was generated in order to determine the relationship between CYP6P9 sequence (FUMOZ-R and FANG) and CYP6P13. CYP6P13 intron was removed so as to compare sequence information of mRNA although no CYP6P13 cDNA amplification was obtained. CYP6P9 from FUMOZ-R and FANG were closely related while CYP6P13 fell on a different branch (Figure 3.8). *Anopheles gambiae* CYP6P3 is a CYP6 gene highly similar to CYP6P9 and was also included in the analysis.

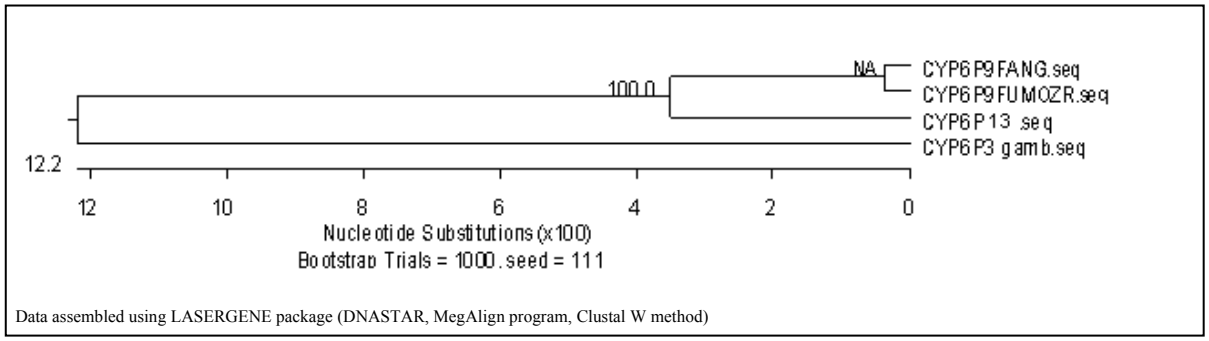


Figure 3.8: Phylogenetic tree of CYP6P9 and CYP6P13. CYP6P9 sequences from FUMOZ-R and FANG were closely related while CYP6P13 sequence was of a different gene. *Anopheles gambiae* CYP6P3 was included as it is very similar to CYP6P9.

3.4.5 Homology modelling of *An. funestus* CYP6P9

Homology modelling was performed so as to visualize the tertiary structure of CYP6P9. The known crystal structure of human microsomal CYP3A4 was used to model putative structures of *An. funestus* CYP6P9. CYP6P9 shares an identity of 26.7% with CYP3A4. The insect CYP6 family is closely related to those of the vertebrate CYP3 family involved in drug metabolism (Danielson *et al*., 1997) hence the use as a model (Figure 3.9A and B). CYP6P9 (FUMOZ-R and FANG) structures

are configurationally the same. Differences are noted on the amino acid within the K-helix (FANG Ile^{361} to FUMOZ-R Met ^{361}) and F-G loop (FANG Ile^{221} to FUMOZ-R Phe ^{221}) (Figure 3.9C and D). The F-G loop is flexible and has been identified as a lid which opens and closes to allow substrates in and out of the active site (Scott *et al.,* 2003; Poulos, 2003).

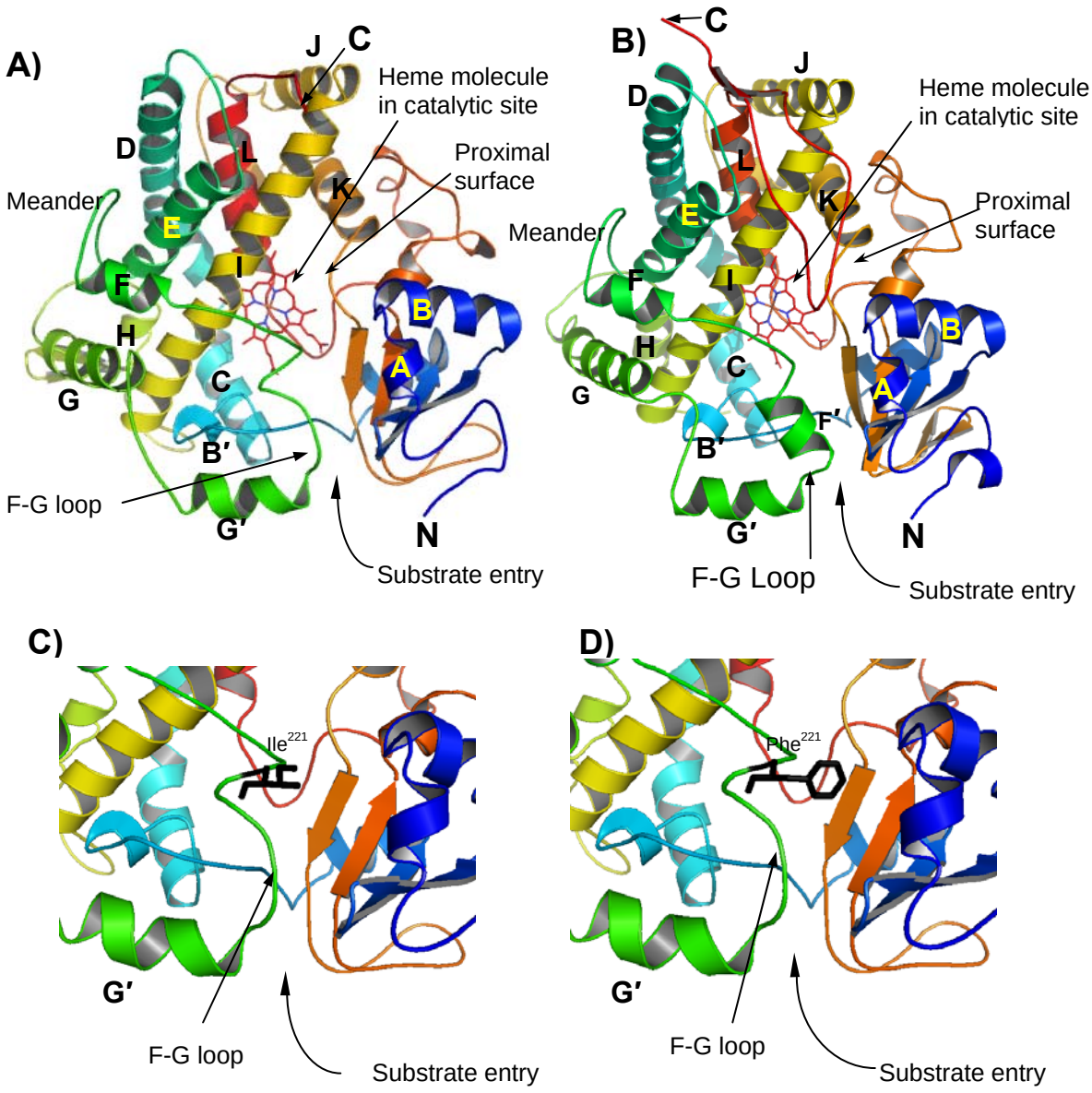


Figure 3.9: Ribbon representation of *An. funestus* CYP6P9s and Human CYP3A4. A) Putative structure of *An. funestus* CYP6P9 B) Crystal structure of Human microsomal P450, CYP3A4, the Heme group (red) is situated within the active site (PDB code: 1tqnA; Yano *et al*., 2004). C) FANG CYP6P9 showing residue ile^{221} within the F-G loop. FUMOZ-R CYP6P9 with Phe^{221} within the F-G loop. CYP6P9 were modeled using known crystal structure of CYP3A4. Substrate entry, catalytic sites and Helices A-L are indicated. N: N-terminal, C: C-terminal. Model was generated using Deep view Swiss-Pdb viewer and PyMOL (Guex and Peitsch, 1997; DeLano, 2002).

3.4.6 Identification of *Anophele funestus* cytochrome b_5 sequence

Anopheles funestus CYP6P9 is a microsomal P450 that requires redox partners for electron transfer from NADPH. As a result of this *An. funestus* redox partners cytb_5 and CPR were isolated. Amplification of *An. funestus* cytb_5 (FUMOZ-R) using *An. gambiae* cytb_5 primers produced a fragment of approximate 400 bp (Figure 3.10). This fragment was cloned into pGEM-T Easy vector in order to sequence and verify whether it belonged to the cytb_5. BLAST analysis revealed the fragment to be 90% identical to *An. gambiae* cytb_5 cDNA. Sequencing results showed that cytb_5 was of 387 bp in size. The start (ATG) and stop (TAA) codons were identified using GeneRunner sequence analysis programme.

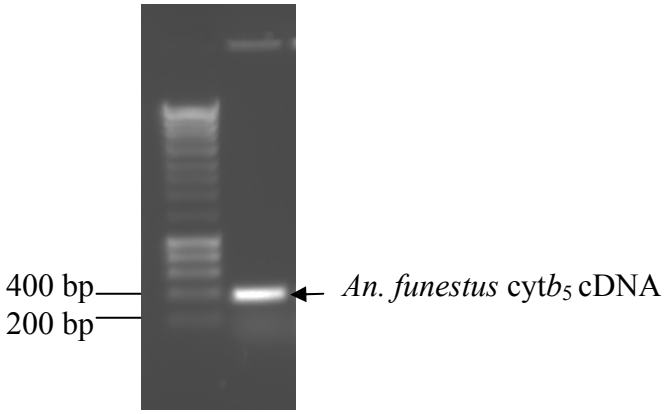


Figure 3.10: Amplification of *An. funestus* cytb_5. *An. funestus* cytb_5 (387 bp) was amplified form *An. funestus* cDNA using *An. gambiae* cytb_5 primers. DNA was run on a 1% TAE agarose gel and viewed under UV light.

The open reading frame of 387 bp encoded a protein of 128 amino acids in length. Sequence alignments revealed high conservation with its counterpart from *An.*

gambiae (Nikou *et al*., 2003) with a percentage identity of 93.8%. As expected cytb_5 sequences from *An. funestus* showed a 59.4% similarity to *M. domestica* (Guzov *et al*., 1996) and a 64.8% similarity to that of *D. melanogaster* (Kula *et al*., 1995) (Table 3.9). Multiple sequence alignment showed that *An. funestus* cytb_5 contains all the conserved amino acids required for the b_5 fold (Trp^{22}, Asp^{31}, His^{39}, Pro^{40}, Gly^{41}, Gly^{42}, Ala^{50}, Gly^{51}, Phe^{58} and His^{63}) (position numbering are based on *Anopheles funestus* cytb_5 sequences) and the two histidines (His^{39} and His^{63}) involved in heme-coordination (Guzov *et al*., 1996; Nikou *et al*., 2003) (Figure 3.11). The amino acids involved in the "b_5 fold" are highly conserved in all sequences. The C-terminus constitutes 67% hydrophobic amino acid residues indicative of the membrane anchor (Vergères and Waskell, 1995).

Table 3.9: Percentage identity amongst various insect cytochrome b_5

	Domcytb_5	**Gamcytb_5**	**Melcytb_5**	**Funcytb_5**
Domcytb_5	-	57.8	76.1	59.4
Gamcytb_5	57.8	-	62.5	93.8
Melcytb_5	76.1	62.5	-	64.8
Funcytb_5	59.4	93.8	64.8	-

Data assembled using LASERGENE package (DNASTAR, MegAlign program, Clustal W method)

```
Domesticacytb5        MSSEDVKYFTRAEVAKNNTKDKNWFIIHNNVYDVTAFLNEHPGGEEVLIEQAGKDATEHF 60
Melanoastercytb5      MSSEETKTFTRAEVAKHNTNKDTWLLIHNNIYDVTAFLNEHPGGEEVLIEQAGKDATENF 60
Gambiaecytb5          MS--EVKTYSLADVKSHNTNKSTWIVIHNDIYDVTEFLNEHPGGEEVLLEQAGREATEAF 58
Funestuscytb5         MS--EVKTYSLAEVKSHNTNKSTWIVIHNDIFDVTEFLNEHPGGEEVLLEQAGKEATEAF 58
                      **  :.* :: *:* .:**:...*::***:::*** ***********:****::*** *

Domesticacytb5        EDVGHSSDAREMMKQYKVGELVAEERSNVPEKSEPTWNTEQK-EESSMKSWLMPFVLGLV 119
Melanoastercytb5      EDVGHSNDARDMMKKYKIGELVESERTSVAQKSEPTWSTEQQTEESSVKSWLVPLVLCLV 120
Gambiaecytb5          EDVGHSSDAREMMKKFKVGELIEAERKQIPVKKEPDWKMDQQ-DDNQLKQWIVPLILGLL 117
Funestuscytb5         EDVGHSSDAREMMKKFKVGELIESGRKQVPVKKEPDWKSEQQ-DDNQLKQWIVPLILGLL 117
                      ******.***:***::*:***: *..:.  *.** *. : *: ::..:*.*::*::***:

Domesticacytb5        ATLIYKFFFGTKSQ 133
Melanoastercytb5      ATLFYKFFFGGAKQ 134
Gambiaecytb5          ATILYRFYFTQ--- 128
Funestuscytb5         ATILYRFYFTQ--- 128
                      **::*:*:*
```

Figure 3.11: Comparison of the open reading frame amino acid sequences of insect cytochrome b_5. Amino acids involved in the "b_5 fold" are indicated in red. The two conserved histidines associated with the heme-coordination are highlighted in bold and underlined. The hydrophobic C-terminal anchor region is represented in grey shade and bold. Identical residues are indicated with asterisks. *An. gambiae*: Gambiaecytb5 (GeneBank accession number: AY183376), *M. domestica*: Domesticacytb5 (GeneBank accession number: L38464), *D. melanoaster*: Melanoastercytb5 (accession number: NP_610294) and *An. funestus*: Funestuscytb5 (GeneBank accession number: EF035450). (ClustalW, http://www.ebi.ac.uk/clustalw/).

Anopheles funestus cytb_5 was modeled using known crystal structure of rat microsomal cytb_5 (Figure 3.12). The two share an identity of 54.3%.

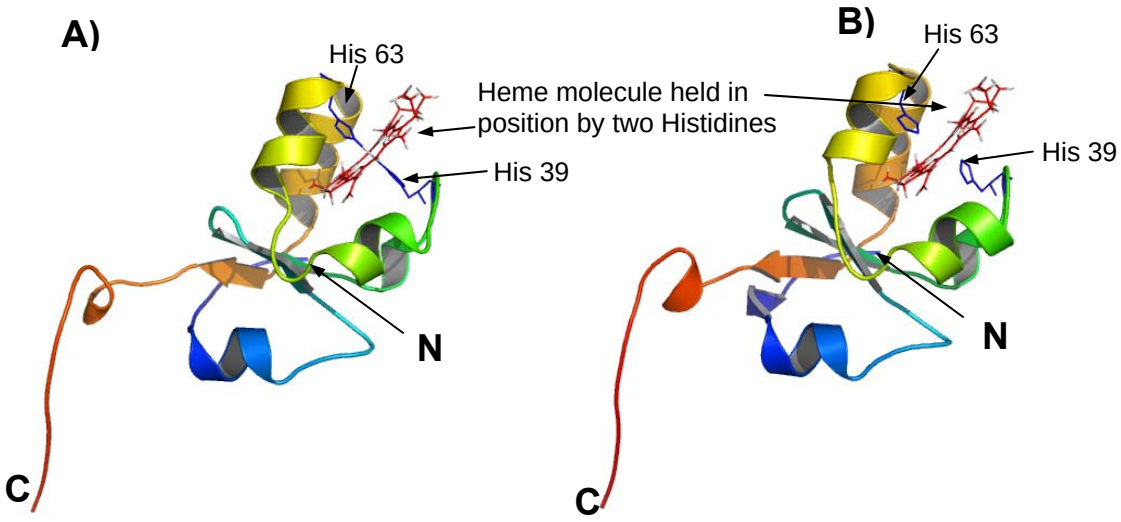


Figure 3.12: Ribbon representation of *An. funestus* and rabbit microsomal cytochrome b_5. A) Crystal structure of rabbit microsomal cytb_5 with heme group (red) (PDB code: 1do9A; Banci *et al.*, 2000). B) Putative structure of *An. funestus* cytb_5. The two Histidine (His^{39} and His^{63}) involved in Heme coordination are shown (Blue). N: N-terminal, C: C-terminal. Model was generated using Deep view Swiss-Pdb viewer and PyMOL (Guex and Peitsch, 1997; DeLano, 2002).

3.4.7 Identification of *Anopheles funestus* NADPH-cytochrome P450 reductase sequence

Anopheles funestus CPR was amplified from cDNA (FUMOZ-R) synthesized from *An. funestus* RNA. A 2000 bp fragment was produced when *An. gambiae* CPR primers were used (Figure 3.13). The fragment was cloned into pGEM-T Easy vector and sequenced. The open reading frame as well as the start (ATG) and stop (TAA) codons were identified using GeneRunner sequence analysis programme. However due to its large size internal primers were designed based on sequences obtained from forward and reverse.

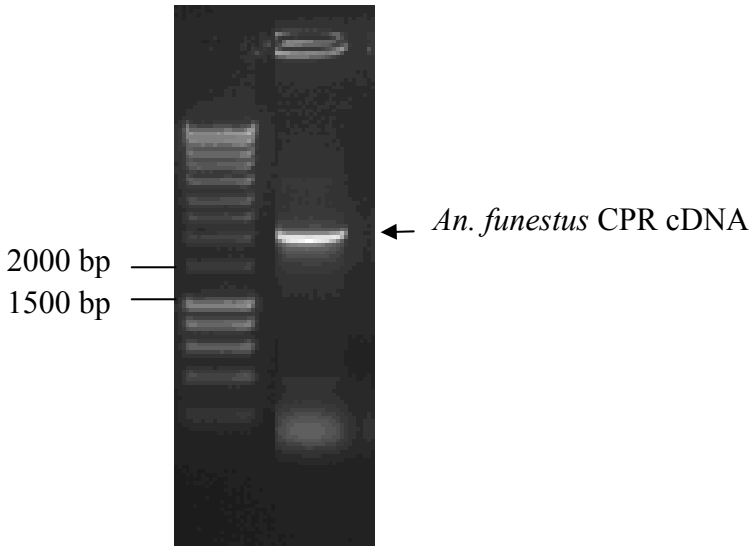


Figure 3.13: Amplification of *An. funestus* CPR. *An. funestus* CPR (2040 bp) was amplified form *An. funestus* cDNA using *An. gambiae* CPR primers. DNA was run on a 1% TAE agarose gel and viewed under UV light.

The *Anopheles* open reading frame of 2040 bp encodes for 679 amino acids. *An funestus* CPR is very closely related to that of *An. gambiae* CPR with a percentage

identity of 96.5% (Table 3.10). A comparison of *An. funestus* CPR with that of *M. domestica* (Koener *et al*., 1993) and *D. melanoaster* (Hovemann *et al*., 1997), revealed highly conserved sequences with identities of 76.3% and 77.5% respectively even though they are evolutionary unrelated (Table 3.10). *Anopheles funestus* CPR contained all conserved regions characteristic of CPRs (Figure 3.14). It had the two conserved tyrosine residues (tyr^{43} and tyr^{181}) (positions are based on *Anopheles funestus* CPR) that are involved in FMN binding as well as the NADPH binding residues Ile^{531}, Phe^{542}, Lys^{625} and Val^{641}. *An. funestus* and *An. gambiae* had two substitutes at positions Arg^{82} and Gly^{188} that were noted within the FMN domain, compared with *M. domestica* and *D. melanoaster* that had Ser and Ala at the same positions. In the FAD domain the only substitution observed was at position Glu^{318} for both *An. funestus* and *An. gambiae* while *M. domestica* and *D. melanoaster* had Asp at this position. A second substitution within the FAD domain was noted in *An. gambiae* at position His^{459} while the other CPRs had Tyr at the same position (Figure 3.14). The N-terminal region constitutes 52% hydrophobic amino acids and is involved in membrane anchoring (Wang *et al*., 1997).

Table 3.10: Percentage identity amongst various insect cytochrome P450 reducatase

	domCPR	**gamCPR**	**melCPR**	**funCPR**
domCPR	-	76.0	84.8	76.3
gamCPR	76.0	-	77.5	96.5
melCPR	84.8	77.5	-	77.5
funCPR	76.3	96.5	77.5	-

Data assembled using LASERGENE package (DNASTAR, MegAlign program, Clustal W method)


Hydrophobic N-terminal anchor region
FMN phosphate moiety
FMN ring (re-face)
FMN ring (si- face)
FAD ring (si-face)
FAD adenine
FAD pyrophosphate
NADPH pyrophosphate

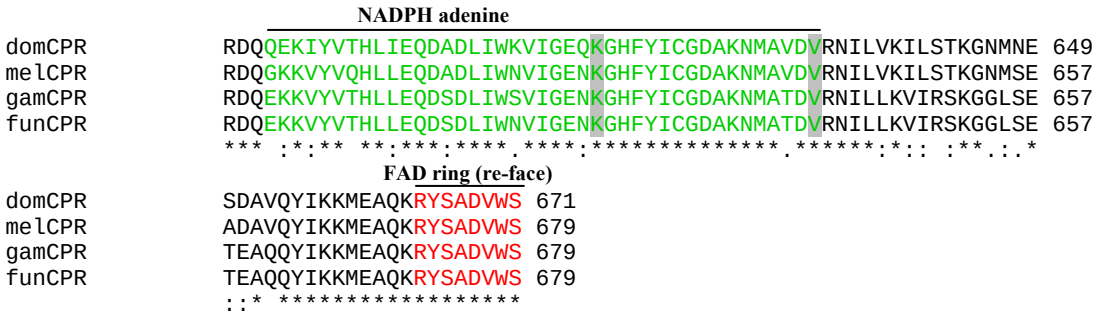


Figure 3.14: Comparison of the open reading frame amino acid sequence of insect NADPH-cytochrome P450 reductase. The FMN binding domain is represented in blue, while the tyrosine residues involved in the FMN binding are shaded in black. The FAD binding domain is shown in red. NADPH binding domain is represented in green and the amino acids involved in the NADPH binding are highlighted by gray shade. The hydrophobic anchor region is indicated in bold. *An. gambiae*: gamCPR (GeneBank accession number: AY183375), *M. domestica*: domCPR (GeneBank accession accession number: L19897), *D. melanoaster*: MelCPR (GeneBank accession number: X93090) and *An. funestus*: FunCPR (GeneBank accession number: EF152578). Identical residues are indicated with asterisks (ClustalW, http://www.ebi.ac.uk/clustalw/).

The putative structure of *An. funestus* CPR was performed using known crystal structure of rat microsomal CPR that had an identity of 56.1% with that of *An. funestus* CPR (Figure 3.15). CPR consists of two main regions, a 6-kDa N-terminal region that is made up of a hydrophobic membrane anchor and FMN domain. A 72-kDa C-terminal region that is composed of the FAD and $NADP^+$ domains making up the catalytic domain. The tertiary structure of CPR is composed of four domains (I-IV) (Figure 3.15).

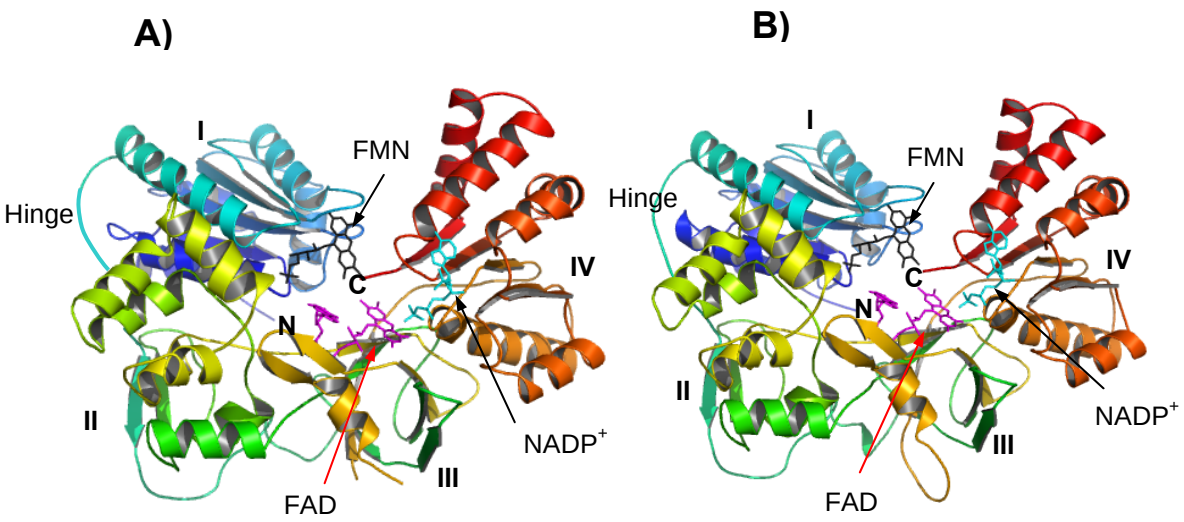


Figure 3.15: Ribbon representation of *An. funestus* and rat CPR. A) Crystal structure of rat CPR, The cofactors are shown as sticks, FMN (gray), FAD (pink) and $NADP^+$ (blue). The four domains are also indicated, FMN domain (I), connecting domain (II), FAD domain (III) and the $NADP^+$ domain (IV) (PDB code: 1amoA; Wang *et al*., 1997). B) Putative structure of *An. funestus* CPR, showing the FMN / FAD and $NADP^+$ binding cleft. N: N-terminal, C: C-terminal. Model was generated using Deep view Swiss-Pdb viewer and PyMOL (Guex and Peitsch, 1997; DeLano, 2002).

3.4.8 CYP6P9 codon usage

The isolated genes were subcloned into a suitable expression vector and proteins expressed in *E. coli* host as seen in the following chapter. Codon usage plays a significant role in the expression of recombinant proteins. Rare codons present in genomic sequences of proteins results in low or no expression. This may be due to early translation termination. Codon usage however determines the selection of host in which the recombinant protein is expressed. The codon usage in *An. funestus* CYP6P9 was analysed. It contained only 18 codons that were rare to *E. coli* (Figure 3.16). This however ruled out the possibility of early translation termination or the

failing to recognize CYP6P9 codons. Table 3.11 shows all the codon used in *An. funsetus* CYP6P9.

```
atggagctaattaacgcggtgttggccgcgttcatcttcgtagtgtcggcagtgtacctt
M  E  L  I  N  A  V  L  A  A  F  I  F  V  V  S  A  V  Y  L
ttcattcggaacaaacataattactggaaagacaatggattcccgtatgcgccgaatccg
F  I  R  N  K  H  N  Y  W  K  D  N  G  F  P  Y  A  P  N  P
cattttctgttcggacacgcgaaaggacaggcccagacaaggcatgcggccgacatccat
H  F  L  F  G  H  A  K  G  Q  A  Q  T  R  H  A  A  D  I  H
ctggaactgtacaaaaaattcaagcagcgcggtgatcggtacgttggtatgagccagttc
L  E  L  Y  K  K  F  K  Q  R  G  D  R  Y  V  G  M  S  Q  F
atcataccatctgtgtttgtgatcgatccagagctggtgaaaacgatcctagtaaaggac
I  I  P  S  V  F  V  I  D  P  E  L  V  K  T  I  L  V  K  D
tttaatgtattccacgatcgcggtattttcactaatgcaagagacgatccactatcagga
F  N  V  F  H  D  R  G  I  F  T  N  A  R  D  D  P  L  S  G
cacttgtttgcgctggaaggtaacccatggcgcttgttgcgtcagaagctcacgccaacg
H  L  F  A  L  E  G  N  P  W  R  L  L  R  Q  K  L  T  P  T
ttcacctcaggtcgcatgaagcaaatgtttggtacattatgggatgtagcacttgagctg
F  T  S  G  R  M  K  Q  M  F  G  T  L  W  D  V  A  L  E  L
gacaagtatatggaagaaaactaccatcagcaggagattgagatgaaggatgtgctaggt
D  K  Y  M  E  E  N  Y  H  Q  Q  E  I  E  M  K  D  V  L  G
cggtttacgaccgatgtgattggcacgtgcgcattcggcatcgagtgtaatacgcttaag
R  F  T  T  D  V  I  G  T  C  A  F  G  I  E  C  N  T  L  K
acaccggactcggaattccgcaaatacggtaacaaagcgttcgagctggatctcctaatt
T  P  D  S  E  F  R  K  Y  G  N  K  A  F  E  L  D  L  L  I
atgatgaagtttttctttgcatcggcttatccgtcacttgtgcggaaactgcgaatgaag
M  M  K  F  F  F  A  S  A  Y  P  S  L  V  R  K  L  R  M  K
atcacattcgatgatgtggaagagtttttcgtaaaaatagtccgcgagacggtgaactat
I  T  F  D  D  V  E  E  F  F  V  K  I  V  R  E  T  V  N  Y
cgtgaaatgaacaatgtaaaacgaaacgacttcatgaacctgctgttgcagatcaagaat
R  E  M  N  N  V  K  R  N  D  F  M  N  L  L  L  Q  I  K  N
aagggcaagctggacgacagtgatgatgggagtgttggcaaaggtgaagtaggaatgaca
K  G  K  L  D  D  S  D  D  G  S  V  G  K  G  E  V  G  M  T
caactggaacttgcggcacaagcgttcgttttcttcctggctggtttcgagacatcatcc
Q  L  E  L  A  A  Q  A  F  V  F  F  L  A  G  F  E  T  S  S
acaactcaaagcttctgtttgtacgagctggcaaagaaccctgaaatccaggagcgcctt
T  T  Q  S  F  C  L  Y  E  L  A  K  N  P  E  I  Q  E  R  L
agacaagagattaaccaagcaatcgaggagaatgacggccaggtgacgtacgatgtcgcc
R  Q  E  I  N  Q  A  I  E  E  N  D  G  Q  V  T  Y  D  V  A
atgaacatacagtatctggacaatgtgataaacgaaacacttcgcaagtacccaccggta
M  N  I  Q  Y  L  D  N  V  I  N  E  T  L  R  K  Y  P  P  V
gaatcgttgagtcgtgtgccgtcggttgactatgtcatccccggtacgaaacacgtgatt
E  S  L  S  R  V  P  S  V  D  Y  V  I  P  G  T  K  H  V  I
gccaaacgaacgttagtgcaaattccggttcacgccattcaacatgatcctgagcactat
A  K  R  T  L  V  Q  I  P  V  H  A  I  Q  H  D  P  E  H  Y
cccgatccagaacgtttcgatccggatcgcttctcaccggaggaagtgaagaagcgacat
P  D  P  E  R  F  D  P  D  R  F  S  P  E  E  V  K  K  R  H
cccttcacgttcctcccattcggcgaggggccacgcgtttgcattgggcttcggtttggt
P  F  T  F  L  P  F  G  E  G  P  R  V  C  I  G  L  R  F  G
gtgatgcagacgaaggtaggattgataacgctgttgagaaagttccgtttctcaccgtca
V  M  Q  T  K  V  G  L  I  T  L  L  R  K  F  R  F  S  P  S
gcgcgtacaccagattgtgtaacgtttcatccgaaaatgatcaccttgtcaccgatcgcg
A  R  T  P  D  C  V  T  F  H  P  K  M  I  T  L  S  P  I  A
ggtaattacttgaaggtggaaaagttgtag
G  N  Y  L  K  V  E  K  L  *
```

Figure 3.16: Codon usage in *Anopheles funestus* CYP6P9. Highlighted (green) are the codons that are rare in *E. coli*. Lue (cta), Arg, (cgg, aga), Ile (ata).

Table 3.11 ***An. funestus*** **CYP6P9 codon usage**

Amino Acid	Codon	Number	/1000	Fraction
Ala	GCG	10.00	19.61	0.37
Ala	GCA	10.00	19.61	0.37
Ala	GCT	1.00	1.96	0.04
Ala	GCC	6.00	11.76	0.22
Cys	TGT	4.00	7.84	0.80
Cys	TGC	1.00	1.96	0.20
Asp	GAT	16.00	31.37	0.52
Asp	GAC	15.00	29.41	0.48
Glu	GAG	17.00	33.33	0.53
Glu	GAA	15.00	29.41	0.47
Phe	TTT	10.00	19.61	0.26
Phe	TTC	29.00	56.86	0.74
Gly	GGG	4.00	7.84	0.15
Gly	GGA	5.00	9.80	0.19
Gly	GGT	13.00	25.49	0.50
Gly	GGC	4.00	7.84	0.15
His	CAT	9.00	17.65	0.69
His	CAC	4.00	7.84	0.31
Ile	ATA	5.00	9.80	0.17
Ile	ATT	15.00	29.41	0.50
Ile	ATC	10.00	19.61	0.33
Lys	AAG	22.00	43.14	0.59
Lys	AAA	15.00	29.41	0.41
Leu	TTG	12.00	23.53	0.25
Leu	TTA	2.00	3.92	0.04
Leu	CTG	15.00	29.41	0.31
Leu	CTA	6.00	11.76	0.13
Leu	CTT	10.00	19.61	0.21
Leu	CTC	3.00	5.88	0.06
Met	ATG	14.00	27.45	1.00
Asn	AAT	11.00	21.57	0.44
Asn	AAC	14.00	27.45	0.56
Pro	CCG	14.00	27.45	0.47
Pro	CCA	10.00	19.61	0.33

Pro	CCT	3.00	5.88	0.10
Pro	CCC	3.00	5.88	0.10
Gln	CAG	13.00	25.49	0.65
Gln	CAA	7.00	13.73	0.35
Arg	AGG	1.00	1.96	0.03
Arg	AGA	3.00	5.88	0.10
Arg	CGG	5.00	9.80	0.17
Arg	CGA	6.00	11.76	0.20
Arg	CGT	6.00	11.76	0.20
Arg	CGC	9.00	17.65	0.30
Ser	AGT	3.00	5.88	0.14
Ser	AGC	3.00	5.88	0.14
Ser	TCG	4.00	7.84	0.19
Ser	TCA	8.00	15.69	0.38
Ser	TCT	1.00	1.96	0.05
Ser	TCC	2.00	3.92	0.10
Thr	ACG	14.00	27.45	0.54
Thr	ACA	9.00	17.65	0.35
Thr	ACT	1.00	1.96	0.04
Thr	ACC	2.00	3.92	0.08
Val	GTG	17.00	33.33	0.49
Val	GTA	9.00	17.65	0.26
Val	GTT	6.00	11.76	0.17
Val	GTC	3.00	5.88	0.09
Trp	TGG	3.00	5.88	1.00
Tyr	TAT	8.00	15.69	0.47
Tyr	TAC	9.00	17.65	0.53
End	TGA	0.00	0.00	0.00
End	TAG	1.00	1.96	1.00
End	TAA	0.00	0.00	0.00

Generatd using http://bioinformatics.org/sms2/codon_usage.html.

Codons in green are rare to *E. coli* and blue are most commonly used codons by *An. funestus* CYP6P9.

3.5 Discussion

The first P450 isolated from *An. funestus* was only recently published (Amenya *et al.,* 2005). Amenya and co-workers isolated 12 CYP6, 12 CYP4 and 6 CYP9 genes which were named by the P450 nomenclature committee (http://drnelson.utmem.edu/CytochromeP450.html). The naming was done using partial fragments of genes. These fragments were further used to identify gene(s) over-expressed in a pyrethroid resistant *An. funestus* strain. One of these, CYP6P9 showed high levels of mRNA expression (Amenya *et al.,* 2008). This work was all conducted on a partial sequence fragment. Preliminary microarray analysis between the pyrethroid resistant and susceptible colonies shows that multiple genes are differentially expressed, including CYP6P9 (Naguran *et al.* unpublished data).

Wondji *et al* (2007) chromosomally mapped a quantitative trait locus (QTL) associated with pyrethroid resistance using the same *An. funestus* FUMOZ-R and FANG strains. They found that a QTL which accounts for more than 60% of variance in susceptibility to permethrin is located between division 9 and 12 of chromosome arm 2R. This location coincides with a cluster of CYP6, P450's mapped by fluorescent *in situ* hybridization, suggesting that resistance is mediated by one or more CYP6 genes. One of these is CYP6P9, an ortholog of CYP6P3 in *An. gambiae*. Apart from this gene's association with resistance nothing else is known about CYP6P9. It is imperative to isolate and sequence CYP6P9 in order to characterize the role of this gene in insecticide resistance.

The high percentage identity between *An. funestus* and *An. gambiae* cytochrome P450s (> 75%) enabled the design of primers for *An. funestus* based on *An. gambiae* sequences (Amenya *et al.,* 2005). CYP6P9 and CYP6P3 show an identity of 86% (Amenya *et al.,* 2005). These primers were used to amplify and isolate open reading frame cDNA sequence of CYP6P9 from *An. funestus* FUMOZ-R and FANG strains as well as genomic sequences. Sequences from these two strains showed a 99.3% amino acid identity. Only four variations were found. Genomic DNA revealed the presence of one intron of 56 bp in size. *An. gambiae* CYP6P3 also had one intron of 56 bp which was found at the same location as *An. funestus* CYP6P9.

When CYP6P9 was compared with other insect CYP6s involved in insecticide resistance it was noted that these sequences shared a very low percentage homology (≤ 45%). The presence of the heme-binding region (PFxxGxxxCxG), ExxR motif, ETLR domain and oxygen binding pocket [(A/G)Gx(E/D)T(T/S)] (characteristics of a cytochrome P450 protein) were identified in CYP6P9; indicating that it was a P450 gene. The characteristic YPDP motif only found in CYP6 families was situated upstream of the heme binding region proving that CYP6P9 belonged to the CYP6 family. CYP6P9 is a microsomal membrane bound protein containing the highly hydrophobic N-terminal region and the PERF motif that is present in all microsomal cytochrome P450s. The evidence is clear, therefore, CYP6P9 belongs to the CYP6 family and that it is a microsomal membrane bound protein.

In FUMOZ-R Ile was replaced with an aromatic Phe within the F-G loop. The F-G loop plays an important role, which operate as a lid in the opening and closing to allow substrates in and out of the active site (Scott *et al.,* 2003; Poulos, 2003). Amino acids found within the F and G helices are important in substrate specificity (Torimoto *et al*., 2007). This was proven in human P450 (CYP3A7) where site directed mutagenesis was performed by changing CYP3A7 amino acid with those of CYP3A4. A decrease in enzyme activity was noted when compared with the CYP3A7 wild-type (Torimoto *et al*., 2007). Mutations found in resistant strains have been shown to give rise to insecticide resistance. This was proven by Bergé *et al* (1998) who suggested that mutations in a CYP6A2 gene from a resistant *Drosopila melanogaster* could affect the tertiary structure, therefore having an important role in the enzyme activity of the protein. It was further proven by Amichot *et al* (2004) who determined that the three mutations outside of the conserved regions of CYP6A2 gene were able to enhance the metabolism of DDT in a resistant strain ($RDDT^R$) as opposed to the wild-type strain which was unable to metabolize DDT. Site-directed mutagenesis in these regions is required to determine the importance of these residues in pyrethroid binding.

A novel P450 gene was isolated from FANG genomic DNA. This gene shared a 93.7% identity with that of FUMOZ-R CYP6P9 genomic DNA. The gene was named CYP6P13 by the P450 Nomenclature committee (http://drnelson.utmem. edu/CytochromeP450. html). Deduced amino acid sequence showed 32 variations between the two genes. When primers were designed based on sequence specific

regions only fragments from FANG genomic DNA were amplified (130 bp and 300 bp fragments). Sequence analysis on the 300 bp fragment revealed that it was identical to CYP6P13 sequence. No fragments were amplified from FANG cDNA or FUMOZ-R genomic DNA and cDNA. This however indicated that CYP6P13 was present at the genomic DNA level of FANG. Phylogenetic analysis showed that CYP6P9 from FUMOZ-R and FANG were closely related. CYP6P13 was on a different branch to CYP6P9 supporting the conclusion that this is a different gene. The absence of CYP6P13 from FUMOZ-R cDNA, FANG cDNA and FUMOZ-R gDNA indicated that the CYP6 gene over-expressed was that of CYP6P9 and not CYP6P13. FUMOZ-R and FANG are from two different geographic locations (Mozambique and Angola respectively). This could explain the presence of CYP6P9 (2) in FANG and an absence in FUMOZ-R. It is likely that a duplication of the gene could have occurred within FANG, however further studies are required to confirm this hypothesis.

The cytochrome P450 redox partners, cytochrome b_5 and NADPH-cytochrome P450 reductase, were also isolated from *An. funestus*. Cytochrome b_5 are membrane bound proteins mainly found within the microsome (Argos and Mathews, 1975). They are 16-kDa globular proteins that contain two domains, a hydrophobic C-terminal membrane anchor and a hydrophilic heme-binding domain where the redox activity takes place (Vergères and Waskell, 1995). They are believed to be responsible for the donation of the second electron to cytochrome P450 during the oxidation of substrates (Vergères and Waskell, 1995; Gruenke *et al*., 1995). The function of *cytb*$_5$

is not clearly understood. The function is dependent on P450 isozymes (Zhang *et al.,* 2005). However high concentrations of cytb_5 have been shown to inhibit the activity of CYP2B4 by blocking P450 reductase binding site (Zhang *et al.,* 2008). To understand the function of cytb5 two substrates were used methoxyflurance and benzphetamine. Cytochrome b_5 was shown to stimulate CYP2B4 resulting in an increase in methoxyflurance metabolism, while the presence of cytb_5 had no change on benzphetamine metabolism (Gruenke *et al.,* 1995; Zhang *et al.,* 2005). *Anopheles funestus* cytb_5 contains the "b_5 fold" and two histidines that are required for heme-coordination (Guzov *et al*., 1996; Nikou *et al*., 2003). The percentage identity of 97% with *An. gambiae* shows that cytb_5 function is conserved between the two species.

Cytochrome P450 reductase has four domains, the FMN domain found near the N-terminus, followed by the connecting domain linking it to the FAD and $NADP^+$ domains at the C-terminus (Wang *et al.,* 1997). Electrons are transferred to the FAD/$NADP^+$ domains before being transferred to cytochrome P450s via the FMN domain (Smith *et al.,* 1994; Wang *et al.,* 1997). CPR donates the first of the two electrons to cytochrome P450 (Vergères and Waskell, 1995). Transfer of electrons to cytochrome P450 is through the FMN domain that is known to bind to the proximal surface of cytochrome P450 (Sevrioukova *et al*., 1999a; Sevrioukova *et al*., 1999b). Cytochrome P450 reductase sequences between *An. funestus* and *An. gambiae* were highly conserved with a percentage identity of 96.5%. Nikou *et al* (2003) showed that CPRs were highly conserved amongst *An. gambiae*, housefly and fruitfly sequences with percentage homologies of above 75%. The FMN and FAD domains,

the conserved NADPH binding residues as well as the two tyrosine residues required for the binding of FMN were also found in *An. funestus* CPR.

Condon usage plays a very important role in heterologous expressing of recombinant proteins in *E. coli*. Some amino acids are encoded by more than one codon. This results in organisms being biased to their own codon. A small number of rare codons within the genes often does not prevent target protein synthesis. However in the presence of clusters of rare codons or numerous rare codons, heterologous expression is very low. Rare codon may result in translational stalling, premature translation termination and translation frame-shifting. Arginine codon (AGG/AGA) is very rarely used in *E. coli* and is known to cause translational problems (Kane, 1995; Kurland and Gallant, 1996). In CYP6P9, 4 of 509 (0.78%) codons are AGG/AGA. The other rare codons are ATA, CTA and CGG. In total there are 18 out of 509 codons that are rare in *E. coli.* Due to the low number and non-clustering of rare codons this may result in very low recombinant protein expression levels when expressed in *E. coli*.

Chapter Four

Protein expression of *An. funestus* CYP6P9 in *E. coli*.

4.1 Introduction

4.1.1 Expression systems for recombinant proteins

Overproduction of recombinant P450 proteins allow for the characterization and understanding of the protein/enzyme activity *in vitro* or *in vivo*. This is used for instance in the characterization of metabolic insecticide resistance or drug design against these resistant enzymes. In order to fully understand P450 enzymes, different host cells are used to express these enzymes in large quantities.

Overproduction of proteins is sometimes problematic and depends on host selection or codon usage. The problem of codon usage arises as a result of low expression or no expression levels in the host. Genes containing rare codons to host cells (e.g. *E. coli,* yeast, insect or mammalian cells) result in translational problems causing a reduction in quality and quantity of synthesized protein (Kane, 1995). When expressing wheat P450 in yeast no expression was obtained due to a high GC content, which is biased to yeast. This problem can be resolved by introducing rare tRNAs or re-engineering the sequence to suit that of the host (Batard, 2000). Various expression systems have been tested such as yeast, bacteria, mammalian cells and insect cells. Each has its limitations as to expressing structural and functional

proteins/enzymes (Battula *et al.,* 1987; Guengerich *et al.,* 1993; Waterman *et al.,* 1995; Grogan *et al*., 1995).

Expression in Escherichia coli (E. coli)

Escherichia coli (*E. coli*) systems are widely used because of their ease and low cost of handling (Waterman *et al.,* 1995). Expression of P450 in an *E. coli* system is mainly achieved by NH_2-terminus modified (Waterman *et al.,* 1995). The first P450 to be successfully expressed in *E. coli* was the bovine microsomal CYP17A (Barnes *et al*., 1991). CYP17A contained a modification by replacing the first eight codons with a short amino acid sequence (MALLLAVF). This short sequence was a derivative of bovine steroid 17 α-hydroxylase that was placed at the NH_2-terminus (Barnes *et al*., 1991).

In order to express an unmodified NH_2-terminal microsomal P450, a technique which involves fusing a bacterial membrane signal peptide to the NH_2-terminal was developed by Pritchard *et al* (1997). Two bacterial membrane signal peptides were used, the *ompA* (outer membrane Protein A) and *pelB* (pectate lyase B) leader sequences. This technique was used successfully to express full-length unmodified human CYP3A4 (Pritchard *et al.,* 1997). To achieve optimum expression the bacterial signal peptide is cleaved upon expression. This is achieved by introducing two amino acids, alanine and proline between the leader sequence and P450 sequence to allow for the efficient removal of this leader sequence. Human CYP3A4 with

fused bacterial membrane signal peptides (*ompA*-3A4 and *pelB*-3A4) had a much higher expression level compared to that with 17 α-hydroxylase modification described above (17α-3A4) (Pritchard *et al.,* 1997). Other P450s such as human CYP2E1 and CYP1A2 as well as *An. gambiae* CYP6Z2 have successfully been expressed in *E. coli* using the *ompA* leader sequence (Dong and Porter, 1996; Döhr *et al.,* 2001; Mclaughlin *et al.,* 2008).

Expression in yeast

The first P450 (Rat CYP1A1) was expressed in Yeast (*Saccharamyces cerevisiae*) (Oeda *et al.,* 1985). Yeast produces its own CPR which acts as electron donor making it a good expression host for P450s. The only limitation is that the endogenous CPR and cytb_5 are insufficient for microsomal P450 activity and there is therefore a need to incorporate exogenous CPR and cytb_5 to aid in expression (Aoyama *et al.,* 1978; Urban *et al.,* 1990; Cheng et al 2006).

Expression in Mammalian cells

Mammalian cells (COS cells) are used to express mammalian P450. These cells are a derivative of the African green monkey kidney cell line (COS- 1 cell) (Zuber *et al.,* 1988). Expression of P450 in COS cells, first requires the insertion into a vector (containing SV40 origin) then transfection into COS-1 or COS-7 cells. After three to four days cells are harvested for activity. The advantage of mammalian P450 expression in mammalian cells is the presence of efficient electron donors (CPR and

cytb_5). No modification is required to the NH_2- terminus cDNA (Reviewed in Gonzalez and Korzekwa, 1995).

Expression in Insect Cells

Expression in insect cells involves using a baculovirus-insect cell expression system. The system has fewer codon usage problems (Duan and Schuler, 2006). While baculovirus is usually restricted to insect cells, many other human P450s have been expressed using this system (Gonzalez and Korzekwa, 1995). The addition of hemin (heme precursor) is required in media during expression of P450 in this system. The advantage of this system for insect P450 expression is that exogenous CPR and cytb_5 are not required. Endogenous CPR is sufficient to achieve high insect P450 activity (Wen *et al.,* 2003). However, expression of human P450 in insect cells requires exogenous CPR electron transfer (Grogan *et al*., 1995; Paine *et al.,* 1996).

4.2 Objective

E. coli will be used as host cells due to its availability. An established yeast or insect cell system was not available at the NICD or the Liverpool school of Tropical Medicine, UK where expression studies were performed. Heterologous expression of microsomal P450 in *E. coli* is extremely difficult without the NH_2-terminal modification. The broad objective of this study was to develop a suitable expression system in *E. coli* to over-produce and purify functional CYP6P9. The following procedures were used in completing this objective. A leader sequence *ompA* was fused to CYP6P9 sequence in order to over-produce the full length enzyme. The

NH_2-terminal modification using 17α hydroxylase was also employed. *Anopheles funestus* cytb_5 and CPR were also expressed in *E. coli.* Plasmid pIX4.0 and pCWori+ plasmid was used to express membrane P450 proteins. Cell induction was achieved by the addition of IPTG and ALA (δ−Aminolevulinic acid) to log-phased cells and expressed protein was analyzed in a spectrophotometer between wavelengths 400-500nm. *Anopheles gambiae* CPR was used instead of *An. funestus* CPR when expression using pCWori+ plasmid was used. This was due to its availability (*An. gambiae* CPR was a kind gift from Dr M.J.I. Paine).

4.3 Materials and Methods

Two vector systems were set up. The first was the newly produced insect vector system (pIX4.0) and the second was the pCWori+. The insect vector was used to express truncated hydrophobic NH_2 terminal P450 enzymes. The pCWori+ vector was used to express NH_2 terminal modified P450 (17αP450) and bacterial signal peptide fused P450 enzymes (*ompA*-P450).

4.3.1 *E. coli* expression of CYP6P9 in insect vector pIX4.0

4.3.1.1 Construction of pIX4.0/$(His)_6$CYP6P9, pIX4.0/$(His)_6$-cytb_5 and pIX4.0/$(His)_6$CPR

Three His-tag constructs were generated for this expression system. Each construct contained restriction sites on NH_2 and COOH terminals to allow for the cloning in to pIX4.0 vector. For the first construct forward primer (5'CTCGAG CCA TCA TCA CCA TCA CCA CAT GGC TGG ACA CGC G 3') (Tm 77.0 ºC) for $(His)_6$-CYP6P9 was designed by replacing the hydrophobic NH_2-terminus (44 amino acids) with *Xho*I restriction site (underline). A six His-tag (red) was inserted prior to the initial methionine (ATG) codon followed by a second codon alanine (GCT) by PCR based mutagenesis (Figure 4.1). The same technique was used for the construction of $(His)_6$-CPR (5' CCATGG ATC ATC ATC ACC ATC ACC ACA TGG CTA AGG GCA AG 3') (Tm 73.7 ºC) except that an *Nco*I restriction site (underline) was added (Figure 4.2). The construction of $(His)_6$-cytb_5 (5'CTCGAG CCA TCA TCA CCA TCA CCA CAT GGC TAA GGG CAA GAA AAA AG 3') (Tm 72.8 ºC) involved the addition of a *Xho*I restriction enzyme site (underline) followed by a 6xHis tag

before the methionine (ATG) start codon and alanine (GCT) as a second codon (Figure 4.3).

Reverse primers for $(His)_6$CYP6P9 and $(His)_6$CPR were designed with a *Bgl*II restriction site (underline) after the stop codons [5'AGATCT CTA CAA CTT TTC CAC C 3') (Tm 58.9 ºC) and 5' AGATCT TTA GCT CCA CAC GCC 3' (Tm 62.6 ºC) respectively], while $(His)_6$-Cytb_5 reverse primer were designed with *Pst*I restriction site (5'CTGCAG CTA CAA CTT TT CCA CC 3') (Tm 62.6 ºC). Amplified cDNAs were ligated into pGEM-T Easy vector as detailed in section 3.3.4. Positive clones were cultured in 5 ml LB broth containing AMP and incubated at 37˚C overnight with shaking. Plasmid DNA was prepared by small scale plasmid isolation. A double digest was performed on plasmid DNA using appropriate restriction enzymes and allowed to digest overnight at 37˚C (Table 4.1).

Table 4.1: Double digestion reaction procedure for DNA

Reagents	**Volume (µl)**	**Concentration**
Water	36	
10x Buffer*	10	2x
DNA	2	(0.5-1 µg/µl)
Enzyme 1	1	0.2 units
Enzyme 2	1	0.2 units
Total volume	**50**	

*Tango Buffer: (33 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA) was used for double digestion (Fermentas, Canada).

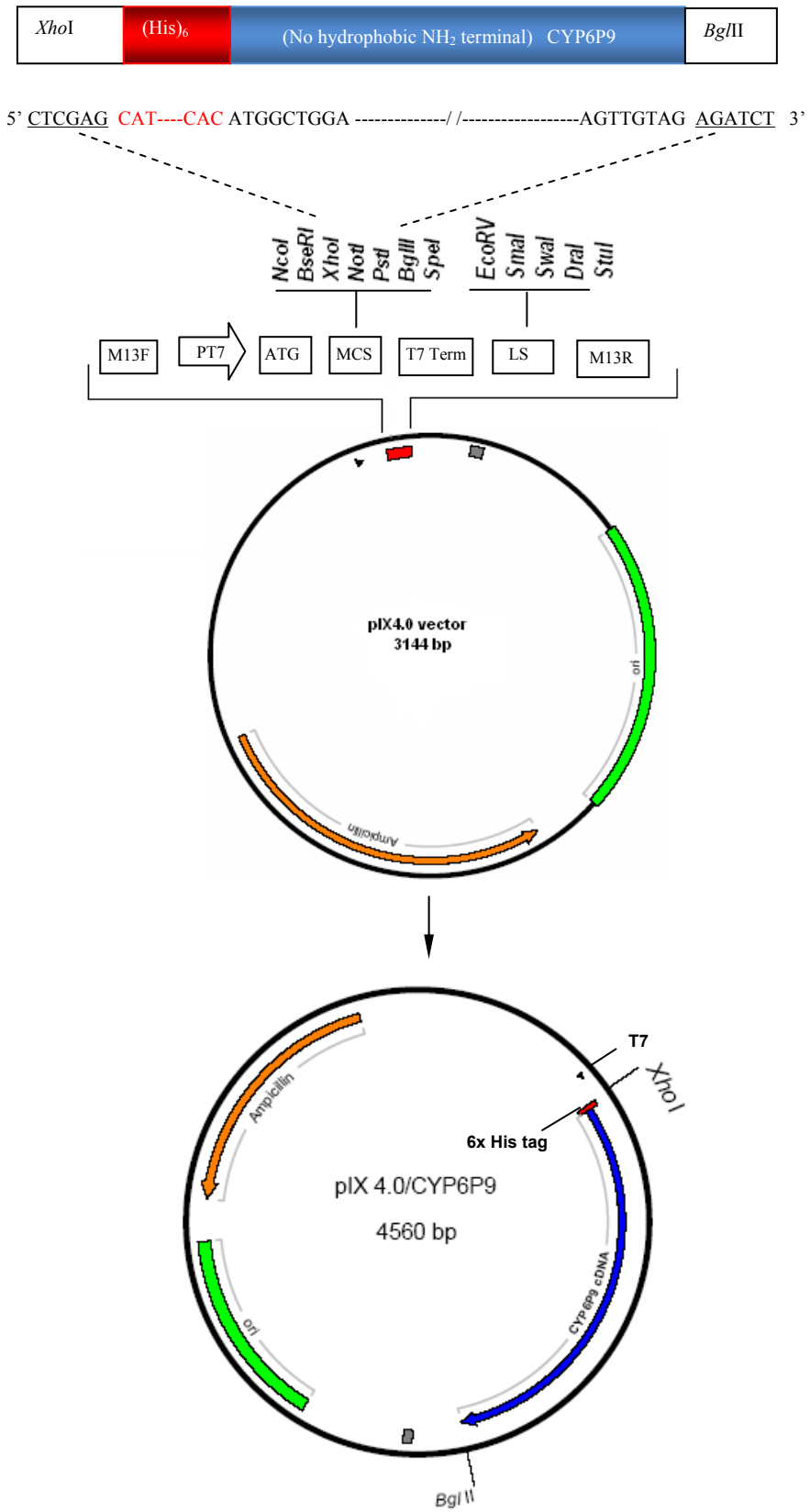


Figure 4.1: Systematic diagram representing the cloning strategy of CYP6P9 into pIX4.0 insect vector. $(His)_6$CYP6P9 was digested from pGEM-T using *Xho*I and *Bgl*II and inserted into a linearized pIX4.0 insect vector to produce pIX4.0/$(His)_6$CYP6P9 plasmid.

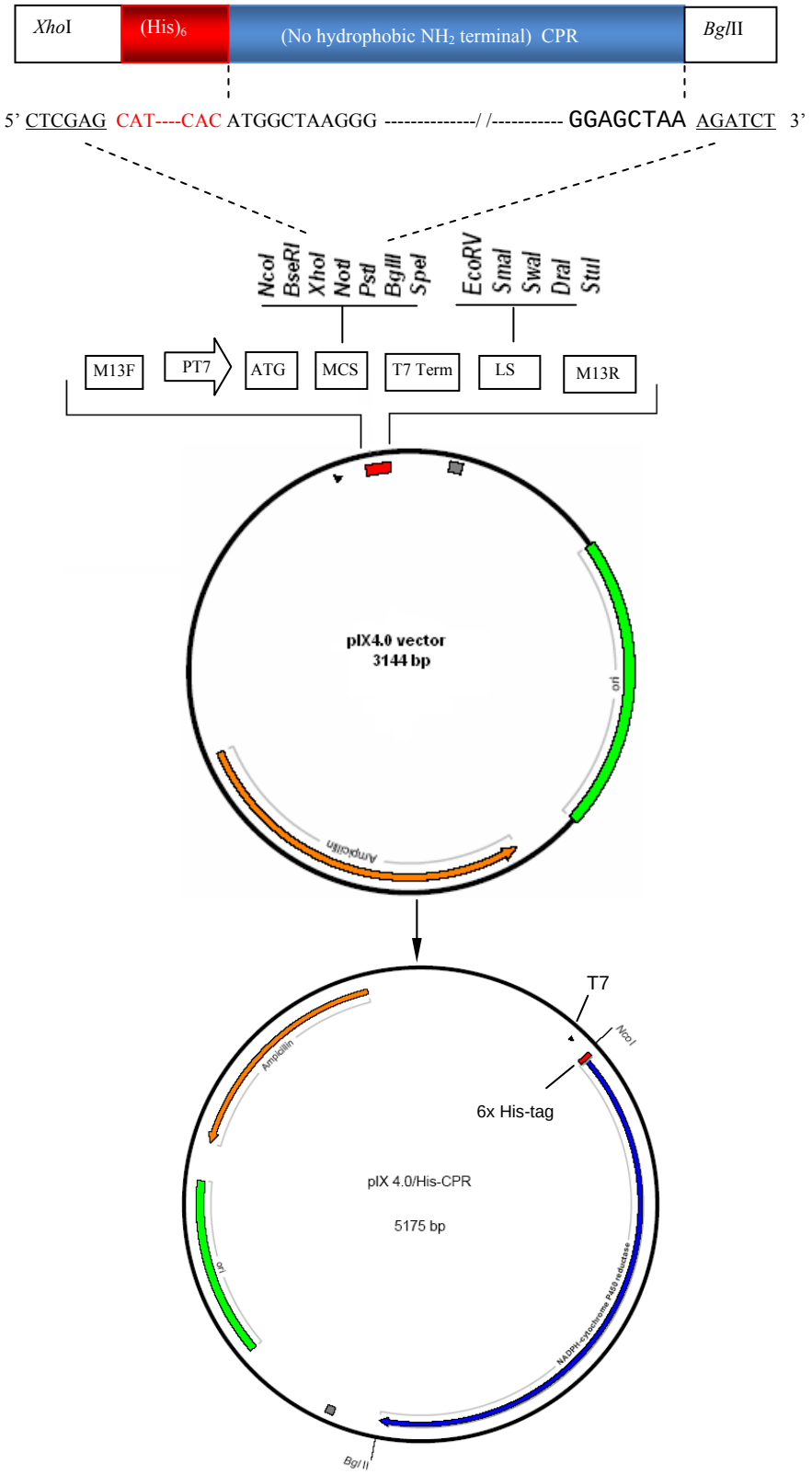


Figure 4.2: Systematic diagram representing the cloning strategy of CPR into pIX4.0 insect vector. $(His)_6$CPR was digested from pGEM-T using *Xho*I and *Bgl*II and inserted into a linearized pIX4.0 insect vector to produce pIX4.0/$(His)_6$CPR plasmid.

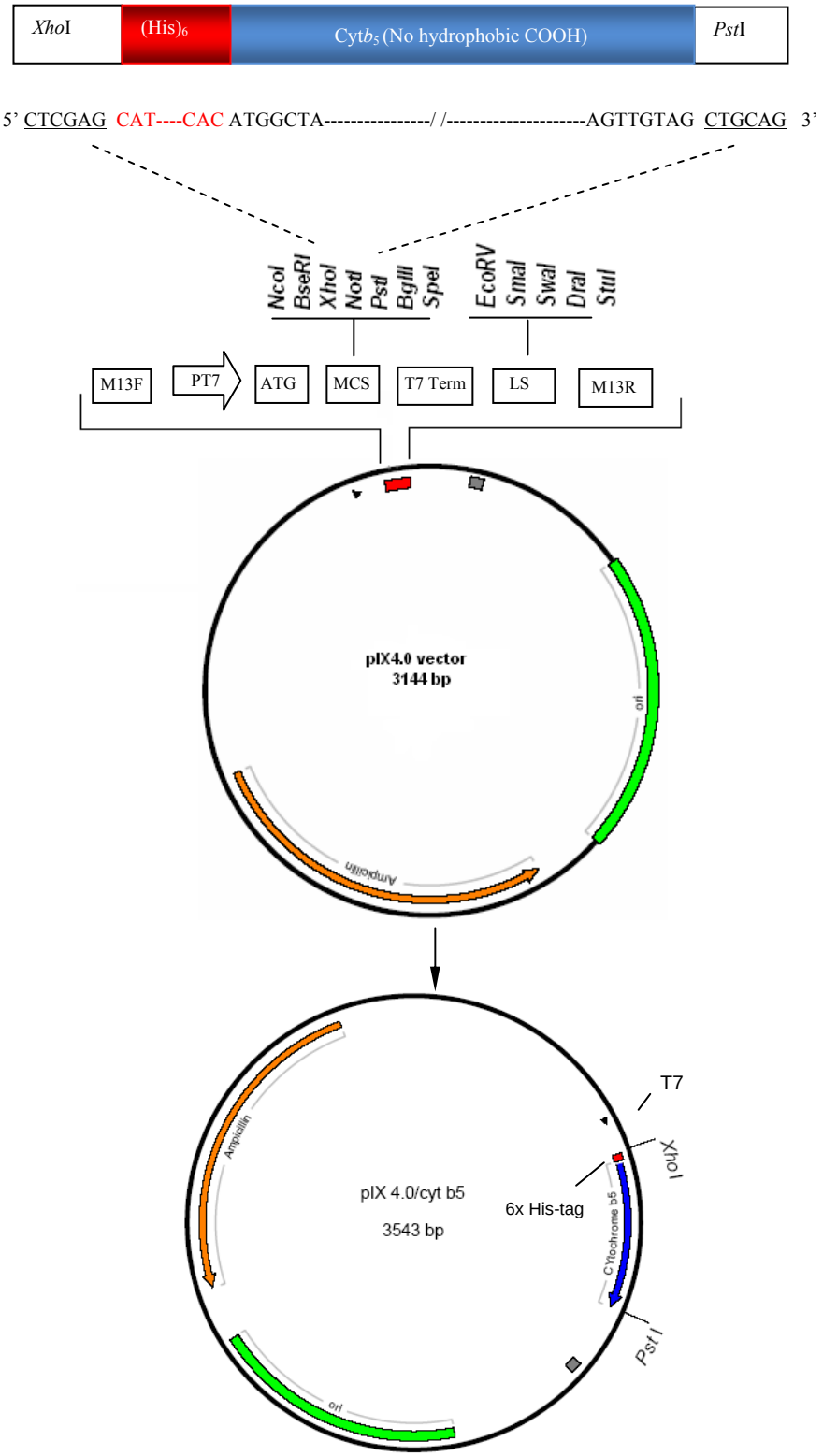


Figure 4.3: Systematic diagram representing the cloning strategy of cytb_5 into pIX4.0 insect vector. (His)$_6$Cytb_5 was digested from pGEM-T using *Xho*I and *Bgl*II and inserted into a linearized pIX4.0 insect vector to produce pIX4.0/(His)$_6$cytb_5 plasmid.

The digested fragments were extracted from 1% TAE (Tris-acetate EDTA) agarose gel and placed into a preweighed 1.5ml microcentrifuge tube. The fragments were purified using the QIAquick gel Extraction Kit (Qiagen, Germany, cat no: 28704). Tube and gel slice were weighed in order to calculate the weight of the gel slice. Three volumes of Buffer QG (Guanidine thiocyanate) were added to the gel (100 mg ~100µL) incubated at 50 ºC until the gel was completely dissolved. Isopropanol (1 gel volume) was added to the sample and mixed. The sample was added to a QIAquick spin column and centrifuged at 16,000 *x g* for 1 minute to bind DNA to the column. Buffer QG (0.5 ml) was added to remove any traces of agarose gel. The sample was centrifuged at 16,000 *x g* for 1 minute followed by one wash with Buffer PE (0.75 ml; containing ethanol) and two spins at 16,000 *x g* for 1 minute.

The QIAquick column was placed into a clean 1.5 ml microcentrifuge tube and 30 µl of ultra pure water was added to the centre of the QIAquick membrane, allowed to stand for 1 minute then centrifuged at 16,000 *x g* for 1 minute. The DNA was stored at -20 ºC until ready for use. The inserts were ligated into insect EasyXpress pIX 4.0 vector (Qiagen, Germany, cat no: 32713) (Table 4.2 and Figure 4.1- 4.3). Transformation and plasmid isolation was performed as detailed in sections 3.3.4 and 3.3.5.

Table 4.2: Ligation procedure for PCR fragment into pIX 4.0 expression vector

Reagents	volume (µl)
2x Rapid Ligation Buffer, T4 DNA Ligase	5
Insect EasyXpress pIX4.0 vector (0.5 µg/µl)	1
PCR Product (200ng)	3
T4 DNA Ligase (3 weiss unit/unit)	1
Total volume	**10**

4.3.1.2 Expression of recombinant P450 proteins

Competent *E. coli* BL21 (DE3) (pLysS) cells were used to transform each of the expression plasmids. Overnight culture (5µl) *E. coli* BL21 (DE3) (pLysS) [transformants] were diluted into 50 ml LB broth containing AMP and CAM. Cultures were grown at 37 ˚C with shaking at 200 rpm until absorbance at 600 nm reached 0.6 - 0.9. Samples (1 ml) were collected from the cultures prior to induction for sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Induction was achieved by the addition of IPTG to a final concentration of 1 mM. Samples (1 ml) were collected from the culture every hour over a period of 5 hours and a final collection after 16 hours. The induced cell pellets were collected by centrifugation at 16,000 *x g* at room temperature for 60 seconds. The cells were resuspended in ice-cold PBS (phosphate buffered saline: 137 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) to a uniform cell density.

A 2x SDS-PAGE gel-loading buffer (100 mM Tris, pH 6.8, 5% β-mercaptoethanol, 4% SDS, 0.2% bromophenol blue and 20% glycerol) was added to each induced cell suspension and boiled for 5 minutes. The tubes were briefly vortexed and 10 µl of

each sample were separated on a discontinuous (0.1%) SDS (12%) PAGE gel electrophoresed in Tris-glycine buffer (pH 8.3) (0.12 M Tris-base, 1.26 M glycine and 0.1% (w/v) SDS) (Table 4.3 and 4.4).

Table 4.3: Preparation of 12% resolving gel

Reagents	Volume (ml)	Final concentration
12% Resolving gel		
Water	4.9	
30% Acrylamide mix (Acryl/ Bis, 29:1)	6	12%
1.5 M Tris (pH 8.8)	3.8	0.38 M
10% SDS	0.15	0.1%
10% ammonium persulphate	0.15	0.1%
TEMED	0.006	
Volume	**50**	

Table 4.4: Preparation of 5% stacking gel

5% Stacking gel		
Water	4.1	
30% Acrylamide mix (Acryl/ Bis, 29:1)	1	5%
1.0 M Tris (pH 6.8)	0.75	0.125%
10% SDS	0.06	0.1%
10% ammonium persulphate	0.06	0.1%
TEMED	0.006	
Volume	**6**	

PageRuler™ Prestained Protein Ladder (mixture of 10 recombinant highly purified proteins with molecular weights from 10 kDa to 170 kDa) (Fermentas, Canada, cat no: SM0671) were used to determine protein size. The gel was electrophoresed at 200V for 45 minutes, stained in gel staining solution (0.24% Coomassie blue, 50%

Methanol, 7.5% Acetic acid) for 3 hours, and destained in destaining solution (20% methanol, 7.5% Acetic acid) overnight.

4.3.1.3 Western analysis detection of His-tagged proteins

Induced cell lysates were run on a (0.1%) SDS (12%) PAGE gel as described in section 4.3.1.2. A PVDF membrane (Polyvinylidene floride) (Millipore, U.S.A cat no: IPVH00010) was placed onto the gel prior to soaking in 100% methanol for 15 seconds and in ultra pure water for 2 minutes (to activate membrane). Gel and PVDF membranes were sandwiched between six layers of 3MM chromatography paper (Whatman, England cat no: 3030917) (three on each side), and two sponges pre-soaked in Transfer buffer (39 mM glycine, 48 mM Tris-base, 20% Methanol). Proteins were allowed to transfer from gel to membrane (towards the anodes) for 1hour at 100 volts in transfer buffer. The membrane was stained in Ponceau-S for 2 minutes at room temperature with shaking then destained in water to confirm and visualize transferred bands.

Western analysis was performed using the HisDetector™ Western Blot kit, HRP Chemiluminescent (KPL, U.S.A cat no: 240002). The membrane was blocked for 1 hour in 1% BSA blocking solution (20 ml; 1% BSA in 1X TBST [Tris buffered saline: 50 mM Tris, 150 mM sodium chloride, pH 7.5, 1% Tween 200]). Then incubated in HisDetector™ Nickel-HRP conjugate (diluted 1/10 000-1/40 000 in 1% BSA blocking solution) for 1 hour at room temperature with gentle agitation. The membrane was washed three times in 1X TBST for 5 minutes each.

Chemiluminescent detection was achieved by applying 5 ml, LumiGLO® working solution (2.5 ml LumiGLO Substrate A and 2.5 ml LumiGLO Substrate B) to the membrane for 1 minute. The substrate was drained and bands were visualized using a G: BOX ChemiHR imaging system (Syngene, UK) for 2 to 10 minutes.

4.3.1.4 Batch purification of recombinant P450 proteins

A culture (50 ml) of *E. coli* BL21 (DE3) (pLysS) [transformants] was inducted as in section 4.3.1.2. Cells were harvested two hours after induction by centrifuging at 4,500 *x g* for 20 minutes at room temperature. The pellet was resuspended in 10 ml native lysis buffer (50 mM sodium phosphate, pH 8.0, with 300 mM NaCl and 10 mM imidazole). Protease inhibitor cocktail (Complete, Mini) (Roche, Germany, cat no: 1836153) (1X concentration) was added to inhibit protease action and lysozyme was also added to a final concentration of 1 mM. The induced cells were lysed by freeze/thawing at -80 °C.

The cells were centrifuged at 4,500 *x g* for 20 minutes at 4 ºC and the supernatant was collected for SDS-PAGE analysis. HIS-Select™ Nickel affinity Gel suspension (1 ml) (Sigma, U.S.A. cat no: P6611) was added to the clear lysate. The mixture was gently agitated at 4 ºC for 1 hour. The lysate- HIS-Select™ Nickel affinity Gel suspension were centrifuged at 4,500 *x g* for 5 minutes in order to remove unbound protein. The supernatant was collected for SDS-PAGE analysis. The lysate- HIS-Select™ Nickel affinity Gel suspension was washed three times in 10 gel volumes of wash buffer (50 mM sodium phosphate, pH 8.0, with 300 mM NaCl and 150 mM imidazole) gently

agitated at 4 ºC. The His-tagged P450 proteins were eluted by adding two gel volumes of elution buffer (50 mM sodium phosphate, pH 8.0, with 300 mM NaCl and 1 M imidazole) then mixing by gently agitated at 4 ºC for 10 minutes followed by centrifuging at 4,500 *x g* for 5 minutes. Elution was repeated four times, collected and analysed by (0.1%) SDS (12%) PAGE.

4.3.2 *E. coli* expression of CYP6P9 in pCWori+ vector

4.3.2.1 Construction of pC-*ompA+2*CYP6P9(His)$_6$ plasmid

i) Construction of CYP6P9(His)$_6$ cDNA

Construction of CYP6P9(His)$_6$ cDNA, forward primer [5' ACCGTAGCGCAG GCCGCGCCG ATG GCT GAG CTA ATT AAC GCG GT 3' (Tm 58 ºC)] with end sequence of *ompA+2* flanking the 5' end (underlined) was designed. Reverse primer [5' GAA TTC TCTAGA TCA GTG GTG GTG GTG GTG GTG CAA CTT TTC CAC CTT CAA GTA ATT AC 3' (Tm 74.3 ºC)] designed with a 6x His-tag (red) before the stop codon and a *Xba*I restriction site (underlined) immediately after the stop codon were used to amplify CYP6P9(His)$_6$. The PCR product was excised from a 1% TAE agarose the gel and the gel purified using the QIAquick gel Extraction Kit as described in section 4.3.1.1. Purified PCR products (5µl) were analyzed on a 1% TAE agarose gel under a UV transilluminator (G: Box ChemiHR imaging system, Syngene, U.K).

ii) Fusion of bacterial leader sequences ompA+2 to CYP6P9(His)$_6$ cDNA

Bacterial membrane leader sequences *ompA+2* (MKKTAIAIAVALAGFATVAQA AP) were synthesized by Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa). In order to fuse *ompA+2* leader sequence to CYP6P9(His)$_6$, a forward primer (P-1)[5' CATATG AAA AAG ACA GCT ATC 3' (Tm 54.7 ºC)] for *ompA+2* was designed with a *Nde*I restriction site (underlined). A reverse primer (P-2) [5' CAC CGC GTT AAT TAG CTC AGC CAT CGGCGCGGCCTGCGCTACGGT 3' (Tm 80.2 ºC)] was designed with the last 21 nucleotide sequences of *ompA+2* (underlined) fused to the first 24 nucleotide sequences of CYP6P9(His)$_6$ cDNA. This produced a PCR intermediate fragment, see Figure 4.4(i) (Pritchard *et al*., 1997). PCR conditions used are shown in Tables 4.5 and 4.6.

Table 4.5: PCR reaction mix for the amplification of intermediate fragment

Reagents	**Volume (µl)**	**Final concentration**
10 x Buffer	5	1x
dNTP mix (10 mM)	5	1 mM
MgCl2 (25 mM)	3	1.5 mM
Primer (1) (3.1 µM)	4	0.248 µM
Primer (2) (3.1 µM)	4	0.248 µM
Water	variable	
DNA	variable	50pg-1µg/50µl
Taq	0.4	0.4 u/µl
Total Volume	**50**	

Table 4.6: PCR cycle conditions for intermediate product

Stage	Cycles	Temperature	Time
1	1	94 ºC	2 minutes
2	30	94 ºC	30 seconds
		55.1 ºC	1 minute
		72 ºC	2.5minutes
3	1	72 ºC	10 minutes

Fusion PCR was used to join and amplify *ompA+2*CYP6P9$(His)_6$ cDNA by mixing the PCR intermediates and CYP6P9 cDNA templates. Forward primer (P-1) and reverse primer (P-3)(5' GAA TTC TCT AGA TCA GTG GTG GTG GTG GTG GTG CAA CTT TTC CAC CTT CAA GTA ATT AC 3') were used to fuse and amplify fragment (Figure 4.4 (ii and iii)(Ninomiya *et al.,* 2004). Each fragment was analyzed on a 1% TAE agarose gel under an UV transilluminator. The reaction mixture is given in Table 4.5 and PCR conditions in Table 4.7.

Table 4.7: PCR conditions for fusion of o*mpA* leader sequence to CYP6P9

Stage	Cycles	Temperature	Time
1	1	94°C	2 minutes
2	30	94°C	30 seconds
		53°C	1 minutes
		68°C	5 minutes
3	1	72°C	7 minutes

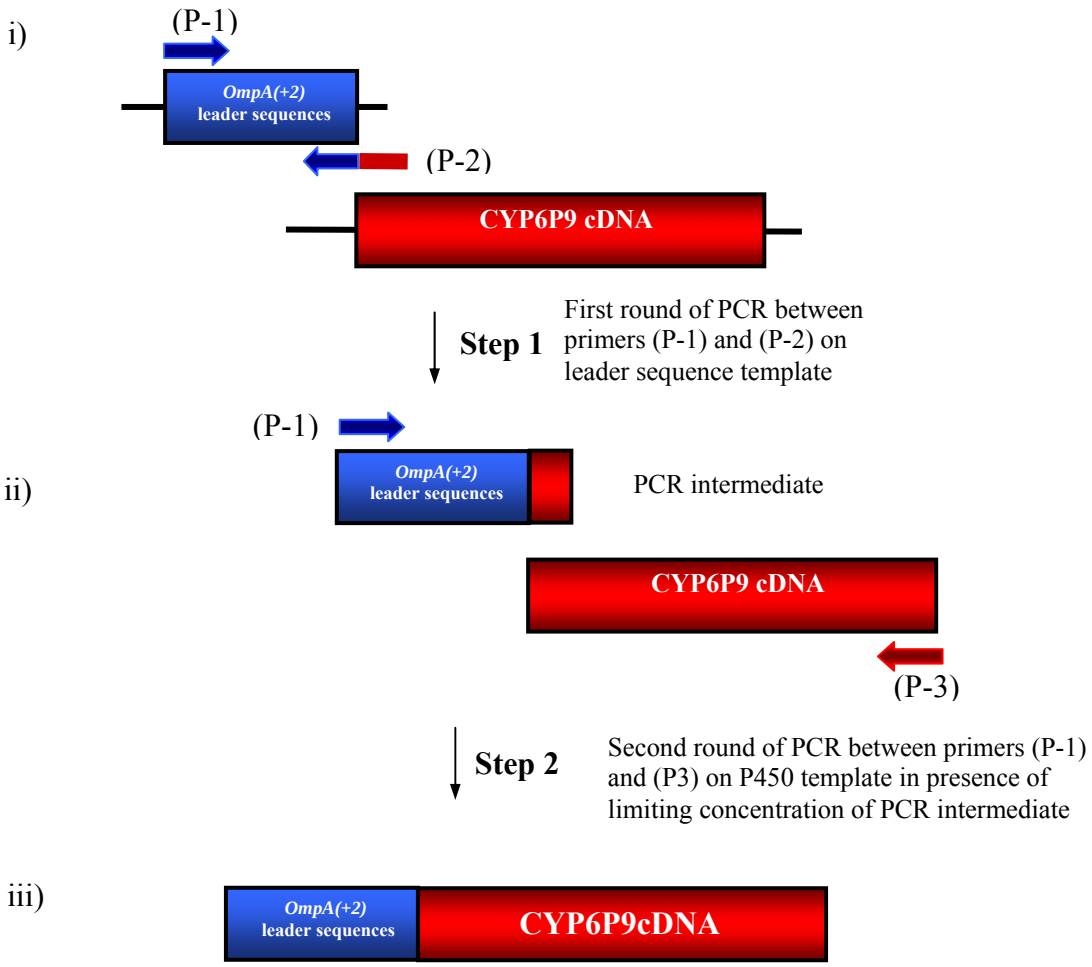


Figure 4.4: Fusion of leader sequences to CYP6P9. i) Primers (P-1) and (P-2) are used to amplify an intermediate fragment that contains a partial sequence of CYP6P9 NH_2-terminus. ii) Intermediate fragment was used as template to amplify and fuse leader sequence to CYP6P9 cDNA using primers (P-1) and (P-3). iii) Leader sequence (*ompA+2*) fused to CYP6P9 cDNA.

iii) Ligation into pCWori+ plasmid

PCR fragment *ompA+2*CYP6P9$(His)_6$ was ligated into pGEM-T Easy vector. Restriction enzymes *Nde*I and *Xba*I were used to excise *ompA+2*CYP6P9$(His)_6$ from pGEM-T Easy vector and prior to ligation into *Nde*I and *Xba*I sites of linearized pCWori+ plasmid (Figure 4.5).

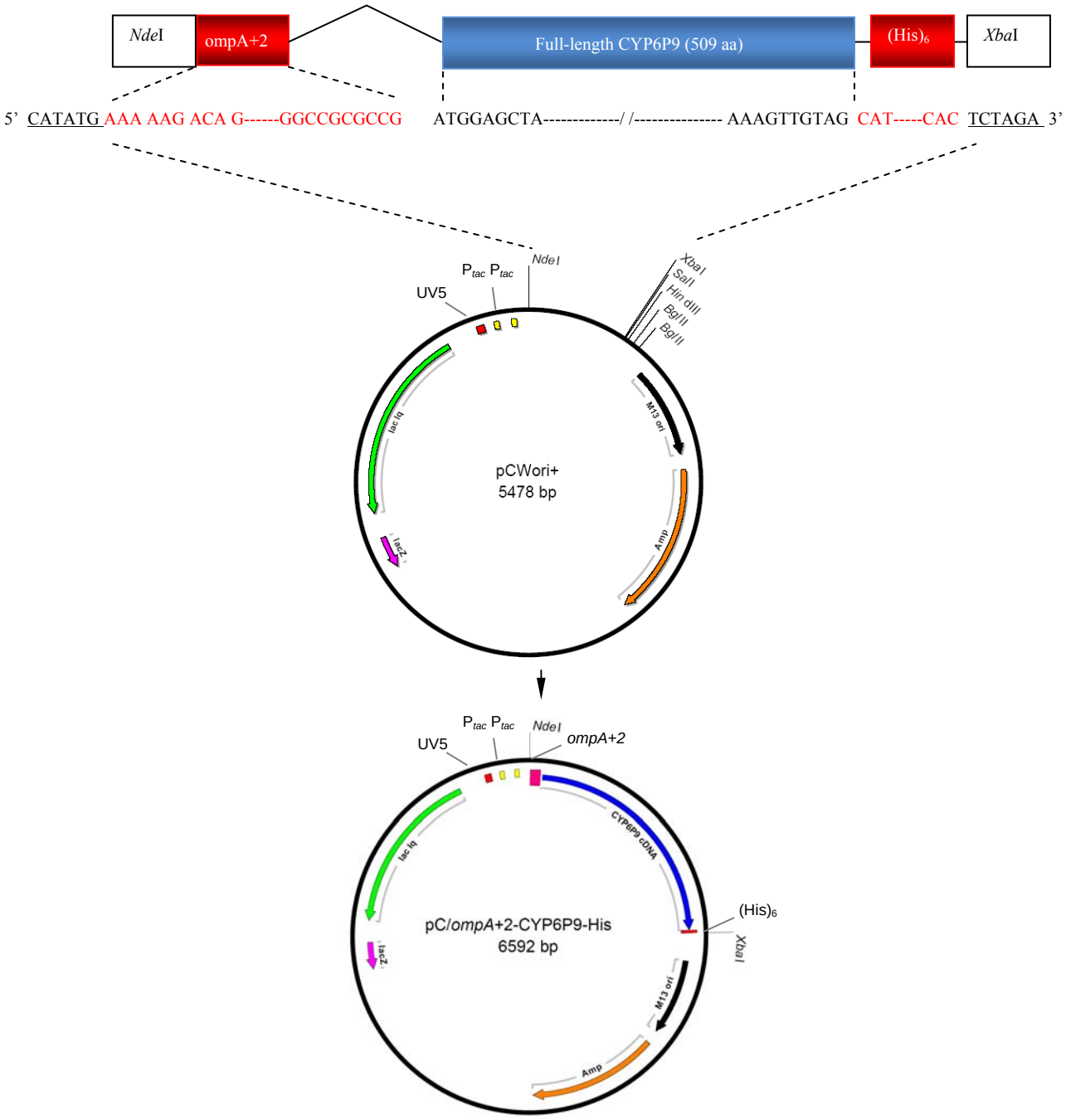


Figure 4.5: Systematic diagram representing the cloning strategy of *ompA+2*CYP6P9(His)$_6$ into pCWori+ expression vector. *ompA+2*CYP6P9 was digested with *Nde*I and *Xba*I and inserted into a linearized pCWori+ vector to produce pC-*ompA+2*CYP6P9(His)$_6$ plasmid.

4.3.2.2 Construction of pCW-*ompA*2CYP6P9 plasmid

i) Amplification of CYP6P9

CYP6P9 was amplified from pGEM-T/CYP6P9 using the forward primer [5' GTAGCCGGCATG GAG CTA ATT AAC GCG GTG 3' (Tm 78.8 ºC)] with *NgoM*VI restriction site underlined before the start codon. The reverse primer [5' ATGAATTCT ACA ACT TTT CCA CCT CAAG 3' (Tm 66.6 ºC)] was designed with *Eco*RI restriction site underlined immediately after the stop codon. KOD HiFi DNA polymerase (Cat No: 71085-3, Novagen, U.S.A) was used to amplify CYP6P9 (Table 4.8 and 4.9).

Table 4.8: PCR mixture for amplification of CYP6P9

Reagents	Volume (µL)	Final concentration
KOD 10x Buffer 1	5	1x
dNTP mix (2 mM)	5	1 mM
$MgCl_2$ (25 mM)	2	1 mM
Forward primer (5 µM)	4	0.4 µM
Reverse primer (2) (5 µM)	4	0.4 µM
Water	variable	
DNA	variable	50pg-1µg/50µl
KOD Polymerase	0.4	0.4 u/µl
Total volume	**50**	

Table 4.9: PCR cycle for the amplification of CYP6P9

Stage	Cycles	Temperature	Time
1	1	96 ºC	5 minutes
2	30	96 ºC	15 seconds
		50 ºC	15 seconds
		72 ºC	1 minutes
3	1	72 ºC	5 minutes
4	1	4 ºC	Hold

The amplified fragment was cleaned using the QIAquick PCR Purification Kit (Cat no: 28104, QIAGEN German). Buffer PBI (5 volumes; containing guanidine hydrochloride and isopropanol) was added to one volume of PCR sample and mixed. A QIAquick column was applied to a vacuum pump. The sample was loaded and allowed to pass through. The DNA was washed with Buffer PE (750 µl; containing 100% ethanol) which was allowed to flow through the sample. The column was transferred into a 2 ml collection tube and centrifuged for 1 minute at 16,000 *x g* then transferred to a clean 1.5 ml centrifuge tube. Buffer EB (30 µL; 10 mM Tris-Cl, 1mM EDTA, pH 8.0) was added to the column which was allowed to stand for 1 minute before centrifuging at 16,000 *x g* to elute the DNA. A three hour digestion at 37 ºC with *NgoM*VI and *Eco*RI was performed (Table 4.10) and digested fragments were excised from a 1% TAE agarose gel run at 100V for 45 minutes. The fragment was gel purified using the QIAquick gel Extraction Kit as described in section 4.3.1.1. Purified PCR fragments (5µl) were analyzed on a 1% TAE agarose gel under a UV transilluminator.

Table 4.10: CYP6P9 cDNA Digestion

Reagents	**Volume (µl)**	**Concentration**
*Eco*RI (12u/µl)	1	0.34
*Ngo*MIV (10u/µl)	1	0.30
BSA (10x)	3.5	1x
NEB buffer4*	0.4	1x
DNA	24.1	
Total	**30**	

*NEB buffer 4: New England BioLabs, U.S.A

ii) Ligation into pCW-ompA2 plasmid

Digested CYP6P9 cDNA fragments were ligated into *NgoM*VI and *Eco*RI sites of the pCW-*ompA2* plasmid (Figure 4.6) (Table 4.11). This plasmid was constructed by inserting the *ompA2* sequence via the *Nde*I site. This site is located down stream of the Ptactac promoter region. An *NgoM*VI site was introduced after the *ompA2* so as to allow ligation of P450 cDNA. The plasmid was also digested with *Eco*RI down stream of the *ompA2* leader sequence site.

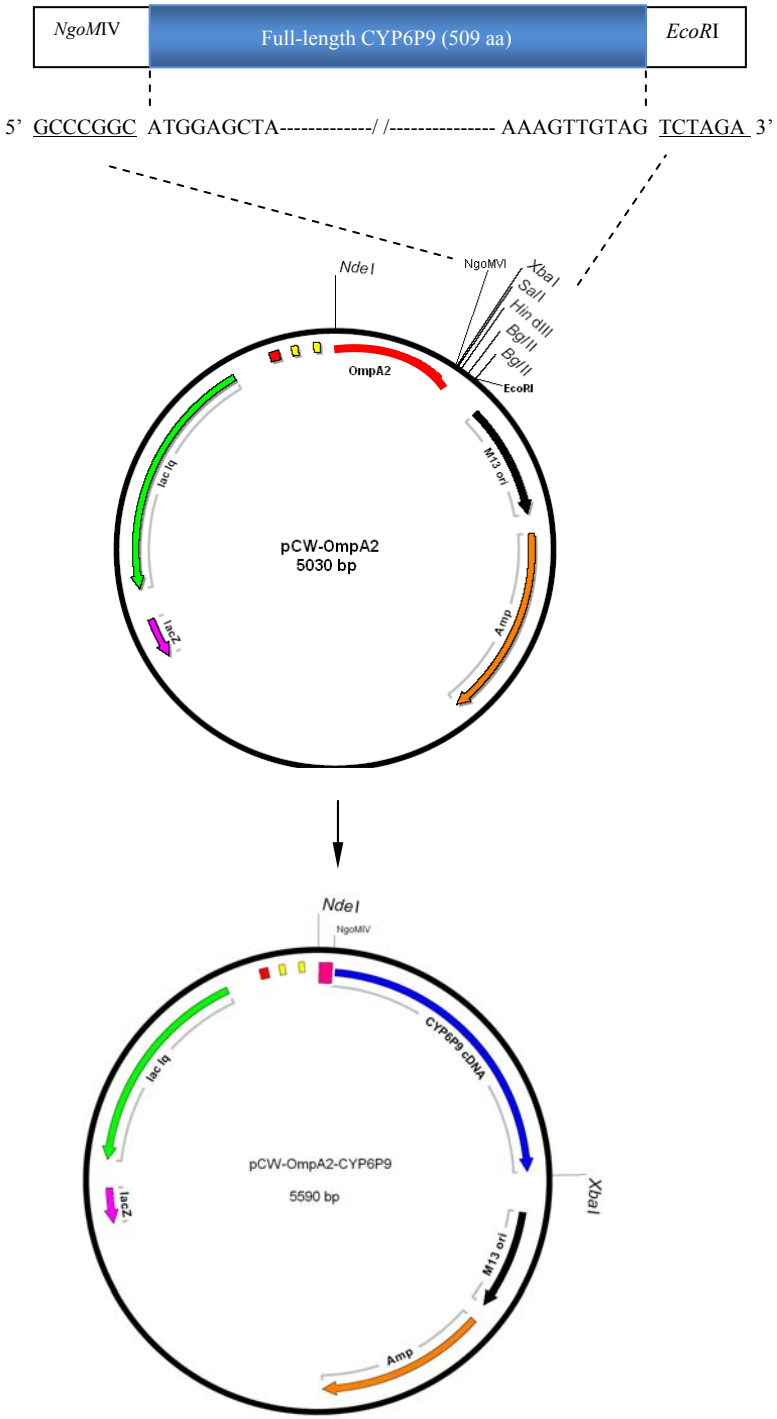


Figure 4.6: Diagram representation of CYP6P9 cDNA insertion. CYP6P9 was digested with *NgoM*IV and *Xba*I and inserted into a linearized pCW-*OmpA*2 vector to produce pCW-*ompA2*CYP6P9 plasmid.

Table 4.11: Ligation into pCW-*ompA2* plasmid

Reagents	volume (µl)
Buffer (10x)	4
pC-*ompA2* plasmid (75 ng/µl)	1
PCR Product (FUMOZ-R: 8.8 ng/ml, FANG: 16.7 ng/µl)	10
T4 DNA Ligase (400,000 unit/ml)*	1
Total	**16**

* New England BioLabs, Cat No: M0202L, U.S.A

iii) Sequence analysis of pCW-ompA2CYP6P9 plasmid

Plasmid, pCW-*ompA*2CYP6P9 was sent to Cardiff University, UK (sequencing facility) for sequencing. Forward primer [5' CCA AGC AAT CGA GGA GAA TGA C 3' (Tm 69.4 ºC)] that recognises part of the pCW plasmid and *ompA2* sequence was used to identify the start sequence of CYP6P9. Reverse primer [5' CAC TCA ATC CCG AAT GCA CAG G 3' (Tm 70.7 ºC)] which recognises the pCW plasmid was used to identify the end sequence of CYP6P9. The internal region was sequenced using the internal forward primer [5' CAA GAG ACG ACC CGC TCT CC 3' (Tm 66.6 ºC)].

4.3.2.3 Construction of pC-17αCYP6P9 plasmid

i) NH_2-terminal (17α hydroxylase) modification of CYP6P9 cDNA

NH_2-terminal modification of CYP6P9 using the 17α hydroxylase sequence was achieved by PCR. 17α hydroxylase sequence replaced the first eight amino acid sequences of CYP6P9 cDNA (Figure 4.7A). Forward primer [5' TTCATATGGCT CTG TTA TTA GCA GTT TTT GC CGC GTT CAT CTT CGT AGT G 3' (Tm

66.5 ºC)] contained *Nde*I site underlined followed by the 17α hydroxylase sequence (MALLLAVF) and 21 nucleotides from CYP6P9 cDNA. Reverse primer [5' CGCTCTAGACTA CAA CTT TTC CAC CT TC 3' (Tm 58.7 ºC)] was designed with *Xba*I restriction site underlined used in the amplification of 17αCYP6P9 cDNA (Figure 4.7B). 17αCYP6P9 cDNA was ligated into *Nde*I and *Xba*I sites of linearized pCW plasmid.

A)

17α hydroxylase	**M**	**A**	**L**	**L**	**L**	**A**	**V**	**F**
	ATG	GCT	CTG	TTA	TTA	GCA	GTT	TTT

B)

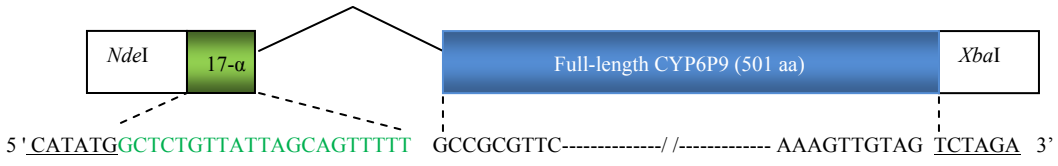


Figure 4.7: 17 α hydroxylase NH_2-terminal modification. A) 17 α hydroxylase sequence, alanine was place as a second codon, silent mutations were introduced to reduce secondary structure. B) Diagram representation of CYP6P9 with 17 α hydroxylase NH_2-terminal modification. Restriction site *Nde*I and *Xba*I were introduced.

4.3.2.4 Transformation into DH5α competent cells

On ice DH5α competent cells (50 µl) (Invitrogen, U.S.A, cat no: 18263012) were transferred into a 1.5 ml centrifuge tube containing DNA (5 µl; 1-10 ng). Cells were gently mixed and stored on ice for 30 minutes. Cells were heat shocked at 42 ºC for 20 seconds in a water bath without shaking. Tubes were placed on ice for 2 minutes

before the addition of SOC media (see appendix A1 for recipe). Cells were incubated at 37 ºC for 1 hour with shaking (225 *rpm*). Transformed cells (200 µl) were spread onto LB plates containing AMP and incubated overnight at 37 ºC.

4.3.2.5 P450 expression in *E. coli*

A starter culture was prepared by picking a single colony from a fresh LB-AMP agar plate and inoculating into 5 ml LB-AMP broth. The culture was allowed to grow overnight at 37 ºC with shaking at 200 rpm. The overnight culture was diluted 1:100 into Terrific Broth (TB) (see appendix A1 for recipe) containing AMP. The culture was grown at 37 °C for 4 hours after which the OD_{600} was monitored until it reached 0.7-1.0. Induction was initiated by the addition of IPTG (1 mM) and ALA (0.5 mM) (δ–Aminolevulinic acid). Induced cells were allowed to grow for 19-24 hours at 25°C.

4.3.2.6 Cytochrome P450 spectral determination

Whole cells (2 ml) were collected and centrifuged at 16,000 *x g* for 2 minutes. The pellet was resuspended in 1X P450 spectrum buffer (2 ml; 100 mM Tris-HCl, pH 7.4, containing 10 mM CHAPS, 20% (v/v) glycerol, and 1 mM EDTA). A few grains of sodium hydrosulfite was added and mixed. The sample was divided equally between a pair of matched 1 ml stoppered optical glass cuvets. A baseline scan from 500 to 400 nm was performed. Samples were bubbled with carbon monoxide (CO) for 45-

60 seconds in a fume hood. A scan from 500 to 400 nm was repeated. An expected peak at 450nm would indicate P450 expression.

4.3.3 Construction of pACYC-*pelB*CPR Plasmid

Plasmid pACYC-*pelB*CPR was a kind gift from Dr M.J.I. Paine (Liverpool school of Tropical Medicine, UK). This plasmid contained *An. gambiae* CPR. *Anopheles gambiae* CPR share an identity of 96.5% with that of *An. funestus.* Due to its availability it was used in place of *An. funestus* CPR in recombinant protein expression. Leader sequence *pelB* was fused to CPR using oligonucleotides containing a *Bam*HI site linked to a *pelB* sequence and 30 bp of the start codon of CPR. The reverse primer contained an *Eco*RI site and 30 bp of the end sequence of CPR. The fragment produced was ligated into *Eco*RI and *Bam*HI sites of pCW plasmid to produce pCW-*pelB*CPR. The *pelB*CPR was cut out of pCW plasmid with the PtacPtac promoter upstream of the *pelB* sequence using *Eco*RV and *Bgl*II. This fragment was ligated into *Eco*RV and *Bam*HI sites of pACYC184 plasmid (*BamH*I and *Eco*RV sites are located within the tetracycline resistant (Tet) gene therefore eliminating the Tet resistance) to produce pACYC-*pel*CPR plasmid (Figure 4.8). pACYC184, does not have an IPTG inducible gene hence the insertion of PtacPtac promoter from pCW plasmid.

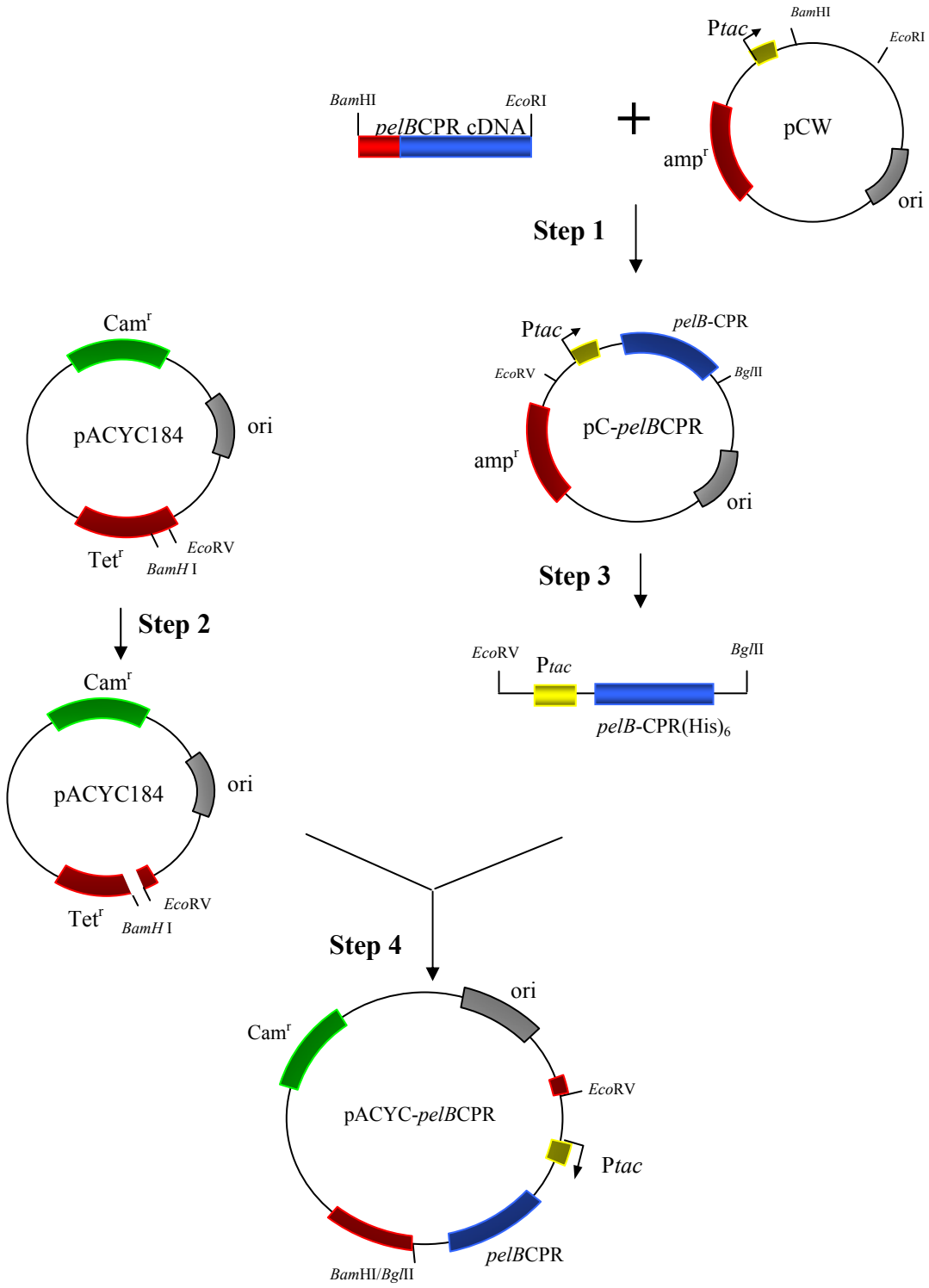


Figure 4.8: Construction of pACYC/*pelB*CPR plasmid. Step1: ligation of *pelB*CPR into pCW plasmid. Step 2: pACYC184 plasmid was linearized with *BamH*I/*Eco*RV. Step 3: *pelB*CPR and P_{tactac} promoter were cut out of pCW plasmid using *Bgl*II and *Eco*RV then ligated into *BamH*I/*Eco*RV site of linearized pACYA184 to produce pACYC/*pelB*CPR (Step 4).

4.3.4 Construction of pC-cytb_5(His)$_6$ plasmid

Construction of cytb_5(His)$_6$ cDNA was carried out using a forward primer [5' CAT ATG GCT TCG GAA GTG AAA ACG TAT TCG CTG G 3' (Tm 69.4 ºC)] that was designed with a *Nde*I restriction site (underlined) flanking the 5' end. Alanine (GCT) that is a preferred second codon by *E. coli* host was engineered after the methionine start codon. The reverse primer [5' GAAGCTTTT AGT GGT GGT GGT GGT GGT GCT GGG TGA AGT AGA ACC 3' (Tm 75.6 ºC)] was designed with a 6x His-tag (red) before the stop codon and a *Hind*III restriction site (underlined) immediately after the stop codon. The amplified fragment was ligated into pGEM-T Easy vector. The insert was cut out using *Nde*I and *Hind*III restriction enzymes and ligated into pCWori+ plasmid linearized using *Nde*I and *Hind*III (Figure 4.9).

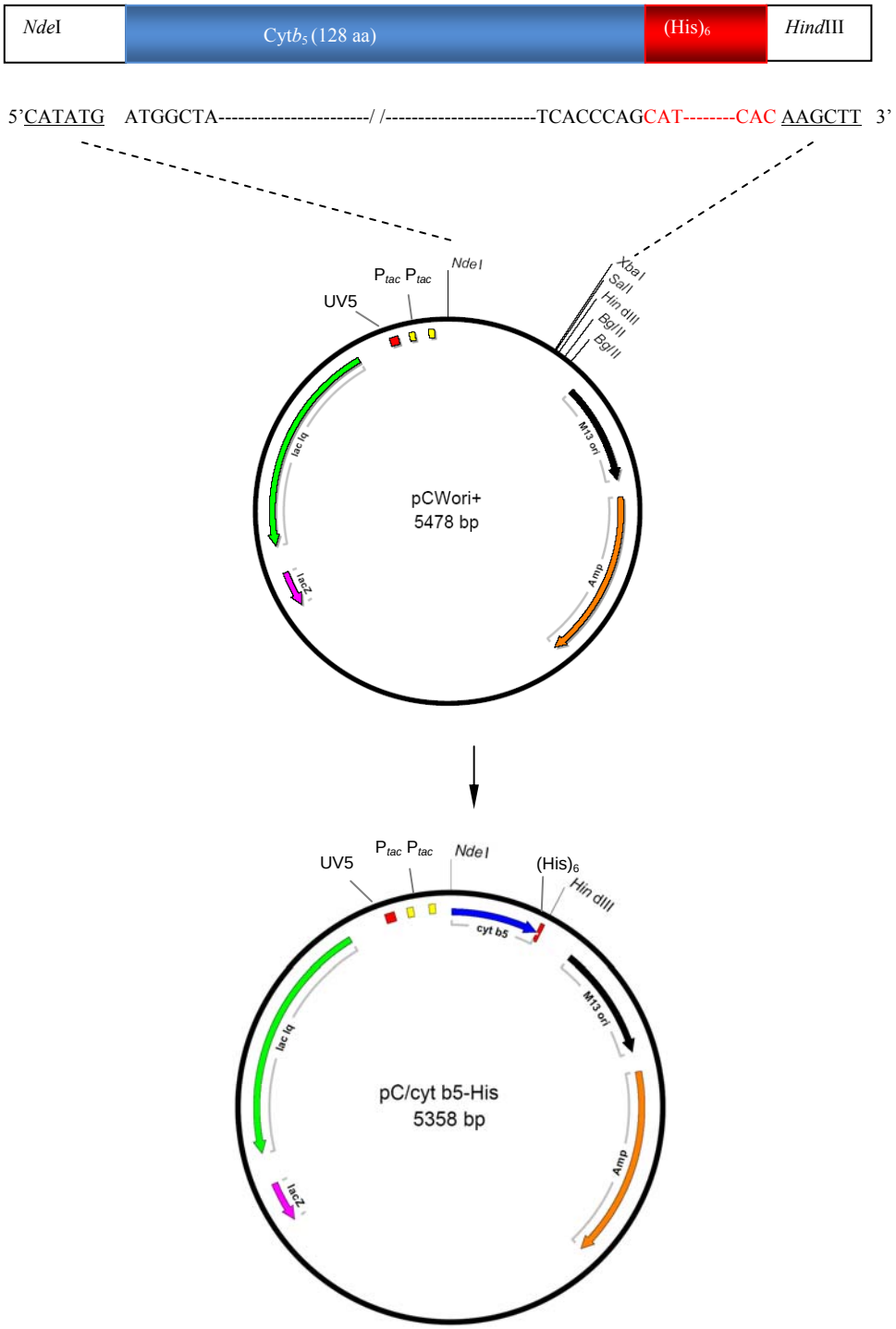


Figure 4.9: Construction of pc/cytb_5(His)$_6$ plasmid. Cytb_5(His)$_6$ was digested with *Nde*I and *Hind*III and inserted into a linearized pCW vector to produce pC/cytb_5 (His)$_6$ plasmid.

4.3.5 Cytochrome b_5 spectral determination

Whole cells (2 ml) were collected and centrifuged at 16,000 *x g* for 2 minutes. The pellet was resuspended in 1X P450 spectrum buffer (2 ml; 100 mM tris-HCl, pH 7.4, containing 10 mM CHAPS, 20% (v/v) glycerol, and 1 mM EDTA). One sample was exposed to air (oxidized) for about 1 hour. A baseline scan from 650 to 350 nm was performed. The oxidized sample was scanned from 650 to 350 nm. A few grains of sodium hydrosulfite was added and mixed (reduced sample) and the scan from 650 to 350nm was repeated to indicate the presence of cytb_5 expression.

4.3.6 Co-expression of CYP6P9 and CPR

Plasmids, pC-*ompA*+2CYP6P9 and pACYC-*pelB*CPR (10 ng each) were transformed into DH5α competent cells as described in section 4.3.2.4. Cells were grown overnight on LB plates containing AMP (resistance conferred to pC-*ompA*+2CYP6P9 plasmid) and CAM (resistance conferred to pACYC-*pelB*CPR plasmid). Positive clones were tested for P450 expression as described in section 4.3.2.7.

4.4 Results

4.4.1 *E. coli* expression of FUMOZ-R $(His)_6$CYP6P9, $(His)_6$cytb$_5$ and $(His)_6$CPR in insect vector pIX4.0

i) Construction of pIX4.0-$(His)_6$CYP6P9, pIX4.0-$(His)_6$-cytb$_5$ and pIX4.0-$(His)_6$CPR plasmids

Isolated FUMOZ-R cDNAs of CYP6P9, cytb_5 and CPR inserted into pGEM-T Easy vector were used to attach a six His tag to the NH_2 terminus of the cDNAs by PCR mutagenesis. Restriction sites *Xho*I and *Bgl*II were introduced to the 5' and 3' ends of $(His)_6$CYP6P9 respectively to give a fragment size of 1,536 bp (Figure 4.10). The fragment was re-cloned into pGEM-T vector. Insect expression vector pIX4.0, containing a bacteriophage T7 promoter which allows for the translation of a cDNA insert was used to express recombinant proteins. Restriction enzymes *Xho*I and *Bgl*II were used to digest $(His)_6$CYP6P9 from pGEM-T (Figure 4.10A) vector and ligated into *Xho*I and *Bgl*II sites of pIX4.0 vector (Figure 4.10B). Plasmid pIX4.0/$(His)_6$CYP6P9 was sequenced and it contained $(His)_6$CYP6P9 cDNA.

*Xho*I and *Nco*I were introduced at the 5' ends of $(His)_6$-cytb_5 and $(His)_6$CPR while *Pst*I and *Bgl*II were inserted at the 3' ends respectively. Fragment sizes of 393 bp and 2046 bp were obtained for $(His)_6$-cytb_5 and $(His)_6$CPR respectively (Figure 4.11A). $(His)_6$-cytb_5 was digested with *Xho*I and *Pst*I from pGEM-T vector and ligated into *Xho*I and *Pst*I sites of pIX4.0 vector (Figure 4.11B), while $(His)_6$CPR was digested with *Xho*I and *Bgl*I (Figure 4.11B). Sequencing was performed on these plasmids and inserts were confirmed.

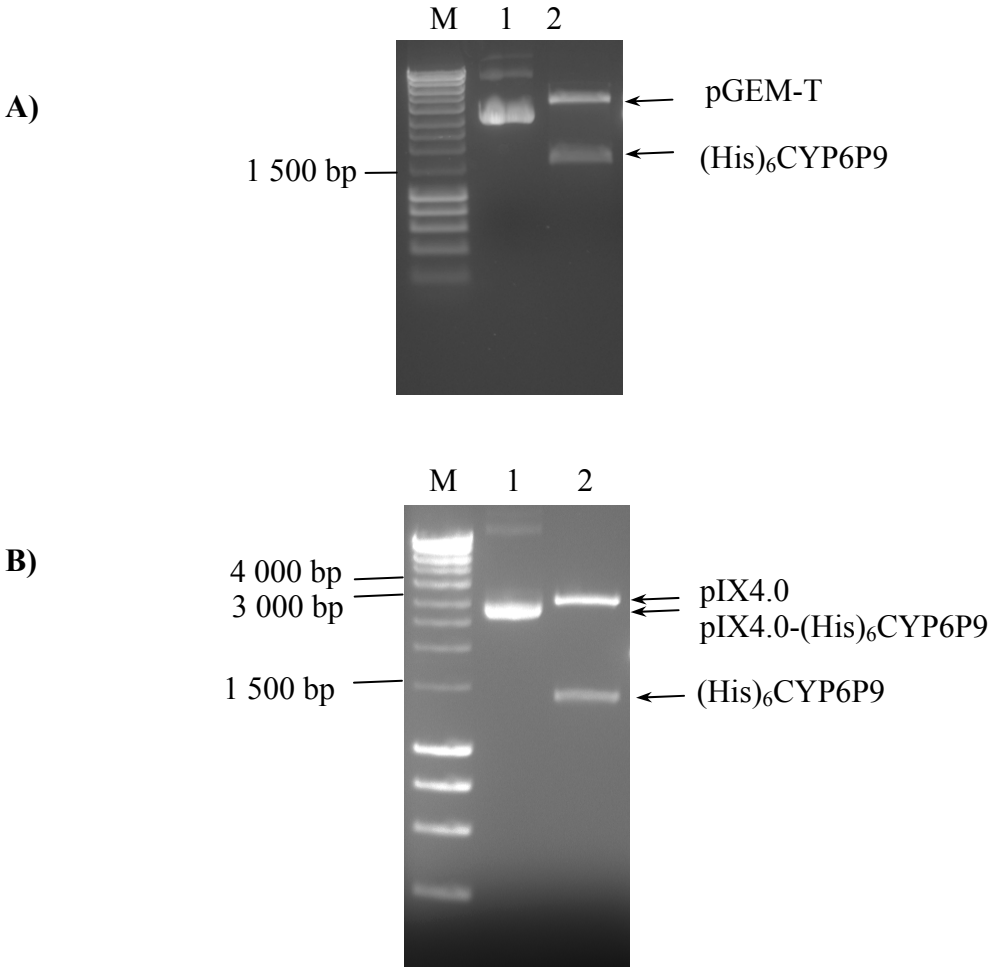


Figure 4.10: Construction of pIX4.0-(His)$_6$CYP6P9 plasmid. A) Lane M: Molecular marker, lane 1: pGEM-T/(His)$_6$CYP6P9 and lane 2: Double digest of pGEM-T-(His)$_6$CYP6P9 using *Xho*I and *Bgl*II restriction enzymes to release (His)$_6$CYP6P9 for subcloning into pIX4.0 vector. B) Lane M: Molecular marker, lane 1: pIX4.0-(His)$_6$CYP6P9 and lane 2: Double digest of pIX 4.0-(His)$_6$CYP6P9 using *Xho*I and *Bgl*II restriction enzymes to confirm (His)$_6$CYP6P9 insert. DNA was electrophoresed on a 1% TAE agarose gel and viewed under UV light.

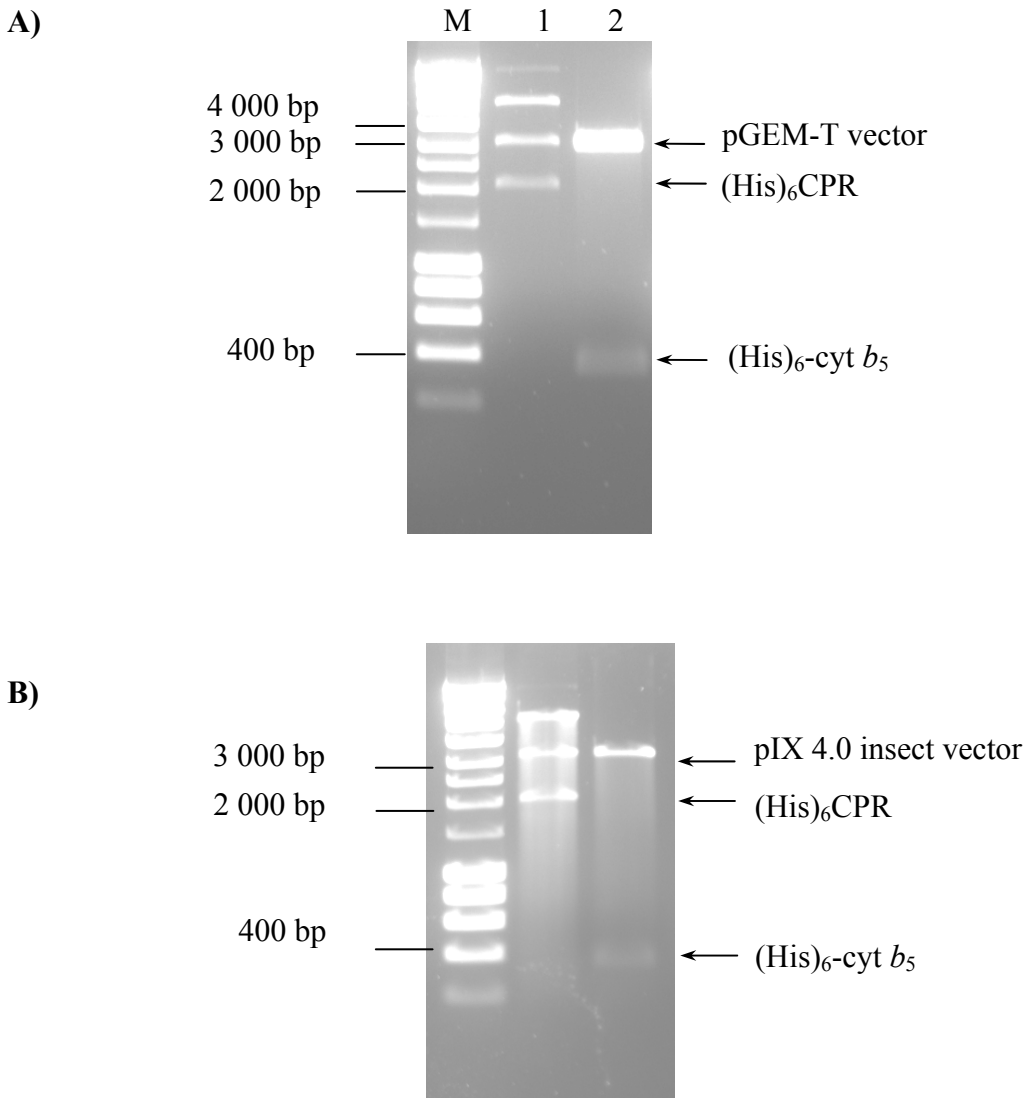


Figure 4.11: Construction of pIX4.0-(His)$_6$cytb_5 and pIX4.0-(His)$_6$CPR plasmids. A) Lane M: Molecular marker, lane 1 Double digestion of pGEM-T/(His)$_6$-cytb_5 using *Xho*I and *Pst*I to release (His)$_6$-cytb_5 and 2: Double digestion of pGEM-T/(His)$_6$CPR using *Nco*I and *Bgl*II to release (His)$_6$CPR. B) Lane M: Molecular marker, lane 1 Double digestion of pIX 4.0-(His)$_6$-cytb_5 using *Xho*I and *Pst*I and lane 2: Double digestion of pIX 4.0-(His)$_6$CPR using *Nco*I and *Bgl*II to confirm (His)$_6$cytb_5 and (His)$_6$CPR inserts respectively. DNA was electrophoresed on a 1% TAE agarose gel and viewed under UV light.

ii) Protein expression of FUMOZ-R $(His)_6$CYP6P9

Protein expression of $(His)_6$CYP6P9 protein in *E. coli* BL21 (DE3) (pLysS) and *E. coli* XL1 Blue proved to be extremely difficult. The hydrophobic N-terminal region was eliminated and a preferred second codon, alanine (GCT) was added after the methionine (ATG) start codon. Induction was initiated with the addition of IPTG (1mM) when the culture reached OD_{600} 0.7-0.9. The first sample (1 ml) was collected just before induction. After induction samples were collected every hour for four hours with a final collection after 16 hours. No expression of $(His)_6$CYP6P9 protein was observed (Figure 4.12). An expected protein size between 50 – 54 kDa was expected for CYP6P9 (Human CYP3A4 an ortholog of insect CYP6 had a size of 54 kDa (Pritchard *et al.,* 1997)). Expression of a protein within this region was observed. To determine whether it was CYP6P9 or bacterial protein a control was included. The control was BL21 (DE3) (pLysS) cell containing no plasmids which was grown in LB broth and induced with IPTG. The profile of the *E. coli* cells were compared with those containing CYP6P9 (Figure 4.12). This clearly showed that protein expressed was that of *E. coli*. Western analysis was performed on induced cells to determine whether CYP6P9 was expressed in low amounts or if there was no expression. The six His tag at the NH_2 terminus is detected by His-antibodies which in turn are detected during Western analysis giving single bands when exposed to an x-ray film or ChemiHR imaging system. No fragments were detected on a Western blot indicating that $(His)_6$CYP6P9 protein was not expressed. Purification of $(His)_6$CYP6P9 was performed using Ni-sepharose beads. His-tag binds to the Nickel beads which are then washed to remove non-specifically bound proteins. Elution is

achieved using imidazole which competed with the His-tag for Nickel binding. A sample of purified protein was run on a SDS PAGE gel and Western analysis was performed to detect the presence of $(His)_6$CYP6P9. No proteins were seen on the SDS PAGE gel and no bands were detected on a Western blot.

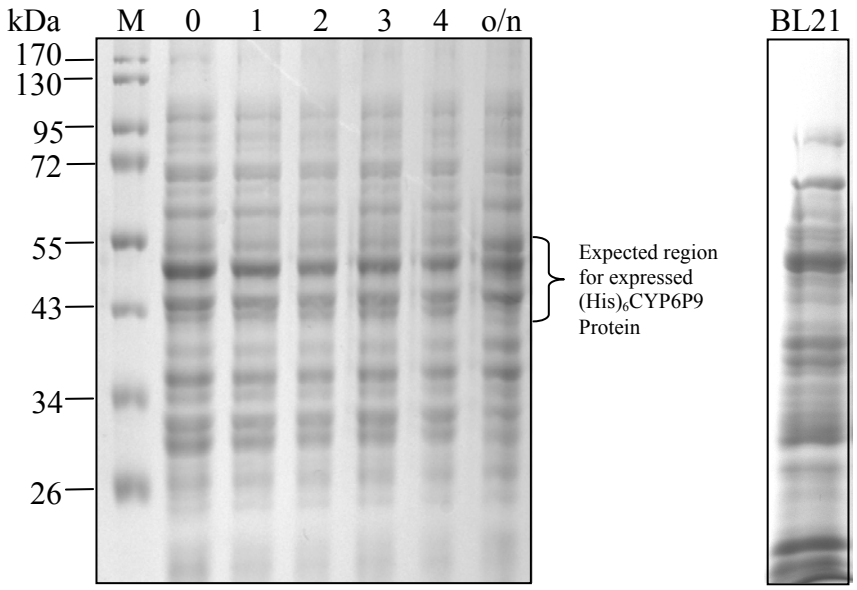


Figure 4.12: SDS-PAGE analysis of $(His)_6$CYP6P9. Lane M: Protein Marker, lane 0: before induction with IPTG, lane 1 to 4: samples collected hourly, 1 to 4 hours after induction with IPTG, lane o/n: overnight (16 hours) sample, lane BL21: *E. coli* BL21 (DE3) (pLysS) protein profile.

iii) Protein expression of FUMOZ-R $(His)_6$ cytb_5 and $(His)_6$CPR

Recombinant expression was performed on $(His)_6$ cytb_5 and $(His)_6$ CPR in *E. coli* BL21 (DE3) (pLysS) and *E. coli* XL1. IPTG was used to induce proteins. No protein bands were noted on SDS PAGE gel (Figure 4.13). The expected protein sizes for $(His)_6$ cytb_5 and $(His)_6$CPR are 19 kDa and 77 kDa respectively. This size

has been shown in other cytb_5 and CPR purified in *E. coli* (Housefly has a size of 19 kDa (Guzov *et al.,* 1996) and *An. minimus* CPR has a size of 77.3 kDa (Kaewpa *et al.,* 2007)). A band was detected in the overnight sample which was in the range of cytb_5. Western blot analysis did not detect this band indicating no expression. Western blot analysis also did not detect any bands for $(His)_6$CPR, hence no expression. Purification of $(His)_6$ cytb_5 and $(His)_6$ CPR also yielded no bands. No $(His)_6$ CPR was detected when inducted and purified.

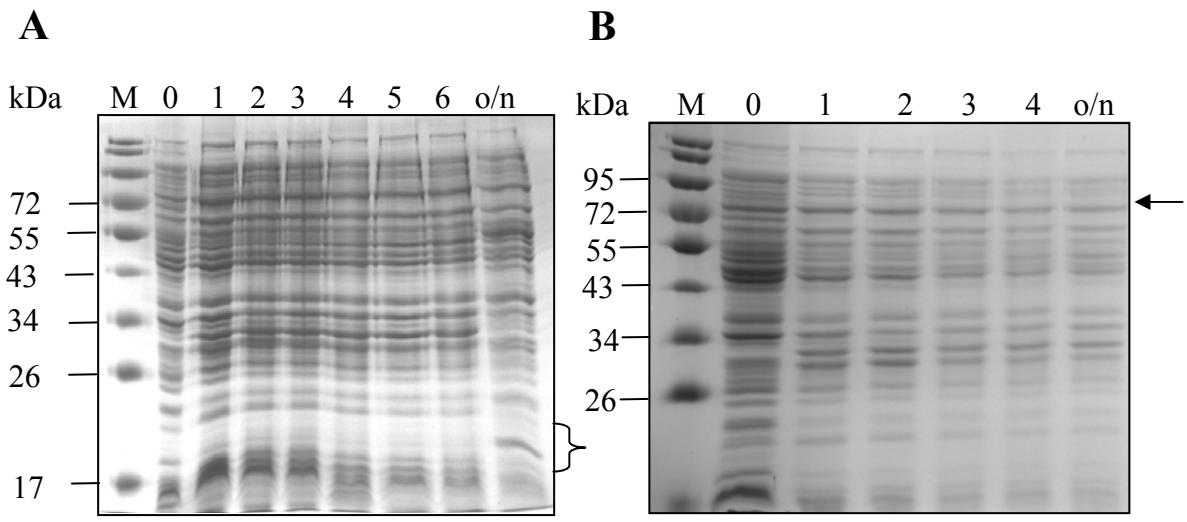


Figure 4.13: SDS-PAGE analysis of $(His)_6$-cytb_5 and $(His)_6$CPR. A) Gel profile for *An. funestus* cytb_5. The expected protein band size of $(His)_6$-cytb_5 is about 19 kDa. Region of expected protein band is indicated by (⌒). Lane M: Protein Marker, lane 0: before induction with IPTG, lane 1 to 6: samples collected hourly, 1 to 6 hours after induction with IPTG, lane o/n: overnight (16 hours) sample. B) Gel profile for *An. funestus* CPR. The expected protein size was about 77 kDa. Arrow indicates position expected for CPR. Lane M: Protein Marker, lane 0: before induction with IPTG, lane 1 to 4: samples collected hourly, 1 to 4 hours after induction with IPTG, lane o/n: overnight (16 hours) sample.

4.4.2 *E. coli* expression of FUMOZ-R CYP6P9 in pCWori+ plasmid

i) Protein expression using bacterial leader sequence (ompA)

Protein expression of P450 proteins in *E. coli* is usually successfully achieved using the pCWori+ vector. The hydrophobic N-terminal region of P450 coding sequence is modified in order to aid in *E. coli* expression. The following experiments were conducted at the Liverpool School of Tropical Medicine in UK where the P450 expression system was available. Protein expression in *E. coli* was monitored using a spectrophotometer between wavelengths 400 and 500 nm. Bacterial leader sequences (membrane signal peptides) *ompA* (Outer Membrane Protein A) was attached to the 5' end of CYP6P9 sequences by fusion PCR technique. To allow for subcloning into pCWori+ vector, *Nde*I restriction site was attached to the 5' end. While a 6x His tag was attached to the 3' end followed by an *Xba*I site to give a fragment size of 1,559 bp (Figure 4.14A). This plasmid construct was referred to as pC-*OmpA*+2CYP6P9$(His)_6$. The third vector tested for expression of CYP6P9 was a pCWori+ that was engineered with an *ompA* leader sequence (referred to as pCW-*ompA*) followed by an *NgoM*IV site to allow insertion of CYP6P9 cDNA. This plasmid was referred to as pCW-*OmpA*2CYP6P9. In contrast to the first construct (CYP6-His_6) no His tag was included into the construct. PCR was used to introduce restriction sites *NgoM*IV and *EcoR*I to 5' and 3' ends of CYP6P9 cDNA respectively (fragment size of 1,537 bp). These restriction sites allowed cloning into pCW-*ompA* vector linearized with *NgoM*IV and *EcoR*I (Figure 4.14B).

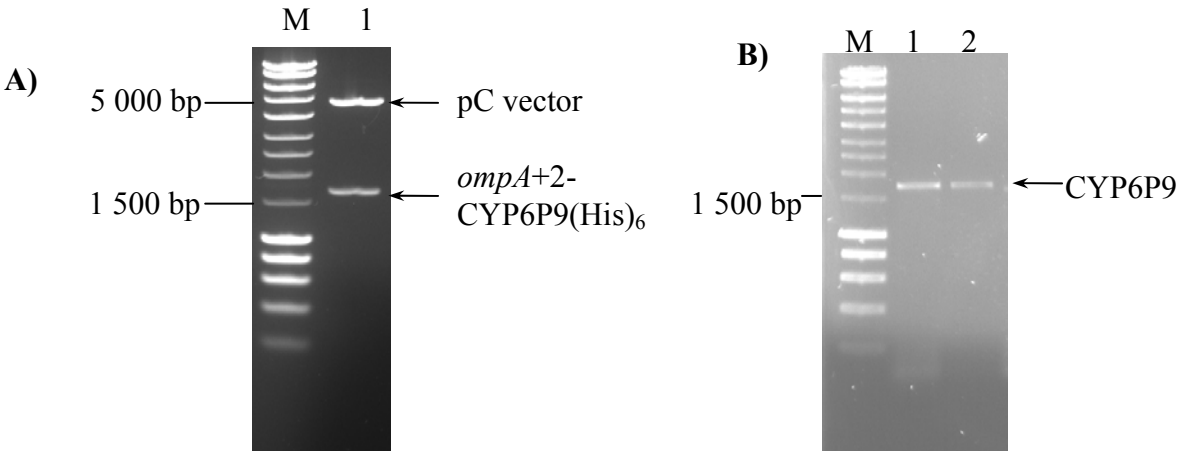


Figure 4.14: Construction of pC-*ompA+2*CYP6P9(His)$_6$ and pC-*ompA2*CYP6P9 plasmids. A) Lane M: Molecular marker, lane 1: Double digest of pC-*ompA+2*CYP6P9(His)$_6$ using *Nde*I and *Xba*I restriction enzymes to confirm *ompA2*CYP6P9(His)$_6$ inserts. B) PCR amplification of CYP6P9. DNA was run on a 1% TAE agarose gel and viewed under UV light.

Protein expression of CYP6P9 proved to be very difficult in *E. coli*, although the preferred *E. coli* expression vector pCWori+ was used. The bacterial leader sequence was attached at the NH_2-terminus of CYP6P9(His)$_6$. Two amino acids proline and alanine were introduced between the *ompA* and P450 gene. Bacterial signal peptidase cleaves the *ompA* and P450 at this site. It is important that *ompA* is cleaved in order to obtain a functional protein. P450 expression is determined by bubbling cell culture (1 ml) with carbon monoxide. The resulting ferrous ion and carbon monoxide complex (Fe^{2+} - CO) forms a peak at 450nm. When CYP6P9(His)$_6$ was expressed in *E. coli,* no expression levels were noted (Figure 4.15). Expression is detected by a peak at 450nm. At least 10 positive clones were selected for expression and they too yielded no expression. When the plasmid was sequenced the *ompA* sequence and

CYP6P9 were in frame ruling out early translational termination. No insertion or deletions were noted within the genes. Plasmid pCW/*ompA*2-CYP6P9 yielded very low expression levels compared to control (*Aedes aegypti* CYP9J28) which had a peak at 450nm indicating high expression (Figure 4.15). No expression was detected when co-expressed with *An. gambiae* CPR or *An. funestus* cytb_5. Protein optimization was performed on both CYP6P9 constructs (pC-*OmpA*+2CYP6P9(His)$_6$ and pCW/*ompA*2-CYP6P9).

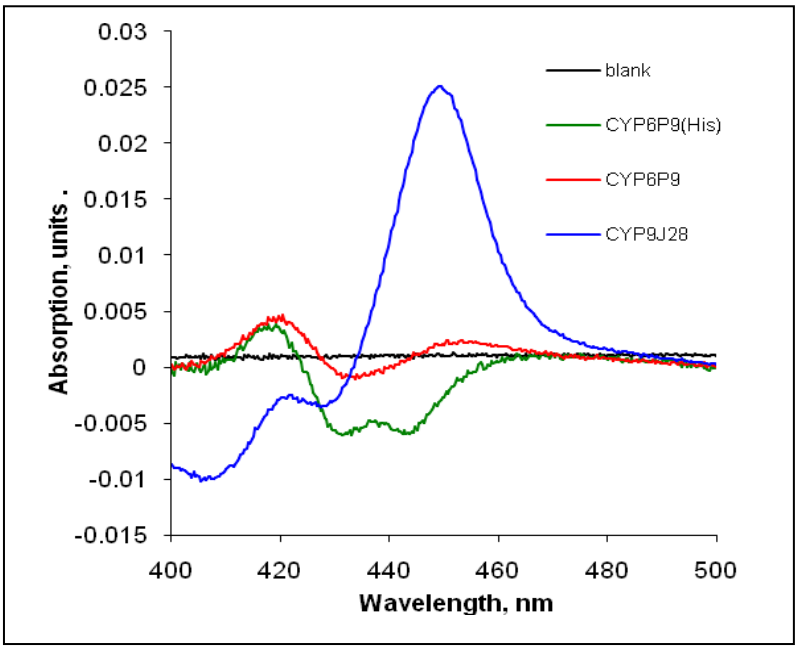


Figure 4.15: CO - difference spectra. CYP9J28 (*Aedes aegypti*) (control) shows an absordant peak at 450 nm indicating P450 expression in *E.coli* (blue line). Expression of CYP6P9(His)$_6$ showing no expression (no peak at 450 nm) (green) and CYP6P9 (no His-tag) showing very low expression at 450 nm (red).

After induction with IPTG and ALA incubation temperatures were reduced to 25 °C, 28 °C and 30 °C to obtain optimum expression. Shaking was kept at 150 *rpm*. Cells were allowed to grow for 3 days. Samples were collected every 24 hours and

randomly within the 3 days. Culture (1 ml) was bubbled with carbon monoxide (CO). P450 expression was measured immediately and also allowed to stand for 10 – 20 minutes to allow Fe^{2+} - CO complex to form.

Growth conditions according to Saribas *et al* (2001) were performed. This involved addition of ALA (0.5 mM) to media after inoculation with overnight culture. Cells were grown at room temperature (25 °C) with shaking at 120 *rpm*. When the cells reached an OD_{600} between 4 -6 they were induced with IPTG (0.7 mM). Cells were allowed to grow for 94 hours. Samples were collected every 24 hours for CYP6P9 expression. With the change in growth conditions no CYP6P9 expression was noted. This led us to try the 17α hydroxylase modification which involved replacing the first eight amino acids with those of MALLLAVF.

ii) Expression using the 17α hydroxylase NH_2-terminal modification

A number of membrane bound P450s have been successfully expressed in *E. coli.* Expression in *E. coli* required the modification of the NH_2-terminus. The first 8 to 18 nucleotides are replaced with the 17α hydroxylase, a derivative of the bovine 17α-hydroxylase P450 (CYP17A). Replacement of the amino acid is predicted to have no effects on protein structure because this region is anchored into the membrane of *E. coli* allowing for CYP6P9 activity to occur on the membrane surface. The sequence consists of eight amino acids (MALLLAVF) which have been shown to improve expression of many P450s in *E. coli*. The second codon was changed to alanine (GCT) however, this is a preferred second codon by *E. coli* for expression of protein.

Silent mutations were introduced to make the codon usage similar to that of *E. coli.* This involved making it AT rich to reduce secondary structure (Figure 4.16A). When the first eight amino acids of CYP6P9 were aligned with those of the 17α hydroxylase, four (50%) of them matched, indicating a similarity (Figure 4.16B). The NH_2-terminus modified CYP6P9 was inserted into pCW vector via the *Nde*I and *Xba*I sites (Figure 4.16C).

A)

Native 17	M	T	L	L	L	A	V	F
	ATG	TGG	CTG	CTC	CTG	GCT	GTC	TTT
Modified 17	M	A	L	L	L	A	V	F
	ATG	**GCT**	**CTG**	**TTA**	**TTA**	**GCA**	**GTT**	**TTT**

B)

```
CYP6P9      MELINAVLAAFIFVVSAVYLFIR--(remainder of CYP6P9 sequence)
17-alpha    MALLLAVF---------------------
            * *: **:
```

C)

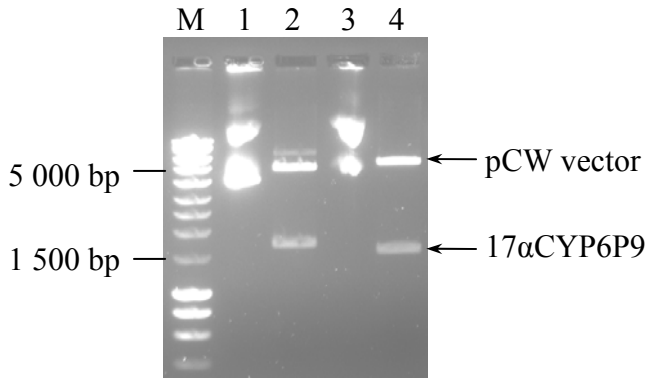


Figure 4.16: Construction of 17αCYP6P9 plasmid. A) Sequence of 17α hydroxylase showing native and modified sequences, silent mutations were introduced so as to reduce secondary structure (red). B) Alignment of CYP6P9 NH_2-terminus with 17α hydroxylase (ClustalW, http://www.ebi.ac.uk /clustalw/). C) Lane M: Molecular marker, lane 1: pCW/*OmpA+2*-CYP6P9$(His)_6$, lane 2: pCW/*OmpA+2*-CYP6P9$(His)_6$ digested with *Nde*I and *Xba*I, lane 3: pCW/17αCYP6P9, lane 4: pCW/17αCYP6P9 digested with *Nde*I and *Xba*I. DNA was run on a 1% TAE agarose gel and viewed under UV light.

Due to the lack of expression detection facilities, the plasmid (17αCYP6P9) was shipped to the Liverpool School of Tropical Medicine, UK to continue with expression studies. Sequence analysis (Cardiff University, Sequencing facility) confirmed the presence of the 17α hydroxylase sequence at the NH_2 terminus. Expression of 17αCYP6P9 recombinant protein was performed in DH5α cells. Cells were grown in Terrific-AMP broth at 37°C. When the culture reached an OD_{600} of about 0.7 the cells were induced with IPTG and ALA. P450 expression is sensitive to temperature therefore the culture media was reduced to 25 °C. Expression of recombinant protein was checked after 24 hours post induction using a spectrophotometer between 400-500 nm. High expression levels were successfully achieved using the 17α hydroxylase approach. No expression was noted in the first 24 hours post induction. Cultures were allowed to continue to grow. Expression was noted on day two with a protein concentration of 1 μM of P450 in the culture. The concentration increased on day three to 2.7 μM of P450 in the culture. A CO-difference spectrum for CYP6P9 was measured over a 2 minute interval after binding with CO. Saturation of CO to CYP6P9 was achieved after 25 minutes (Figure 4.17). This however was in contrast to *An. gambiae's* CYP6M2 and CYP6Z2 recombinant proteins which are saturated after three minutes.

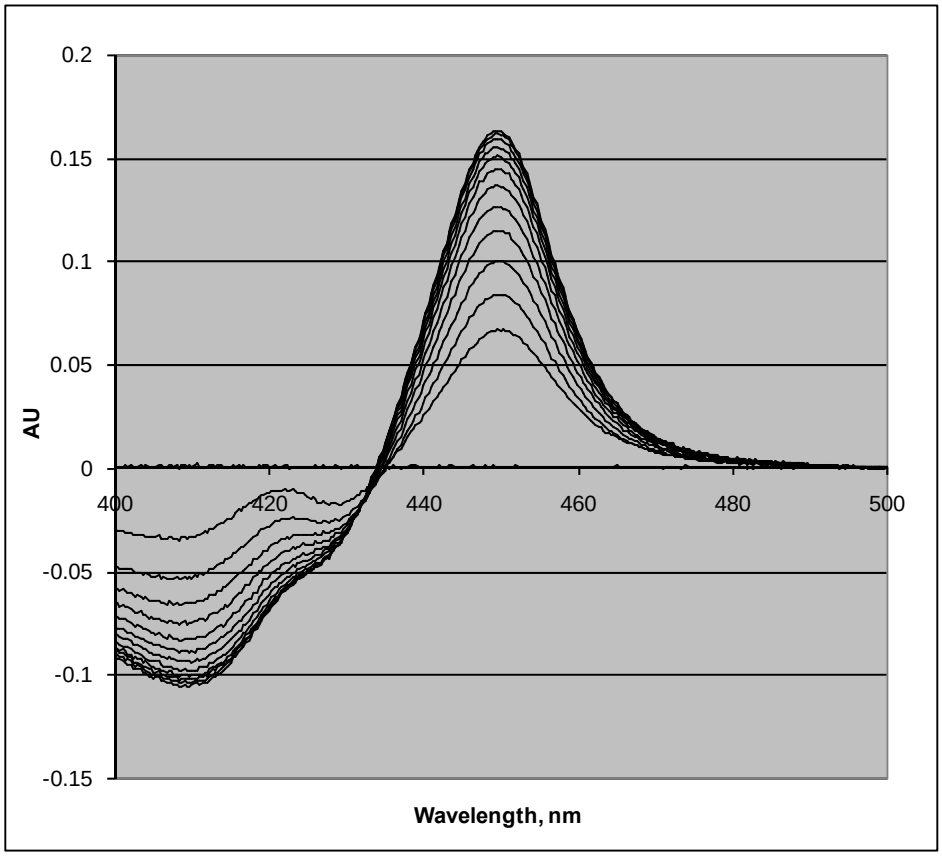


Figure 4.17: CO - difference spectra. Expression of 17αCYP6P9 was achieved after three days. Peaks represent measurements carried out every two minutes until a maximum (CO-P450 saturation) was achieved after 25 minutes. AU-absorption units. (expression was performed courtesy of Dr Bradley Stevenson, Liverpool School of Tropical Medicine, UK).

4.4.3 Expression of *An. funestus* cytb_5 in *E. coli*

Cytb_5 has a C-terminal hydrophobic region hence it does not require a bacterial leader sequence for expression. Construction of pC/cytb_5(His)$_6$ involved the insertion of *Nde*I to the 5' end and a 6x His tag followed by a *Hind*III at the 3' end of Cytb_5. The fragment was ligated into *Nde*I / *Hind*III linearized pCWori+ vector (Figure 4.18).

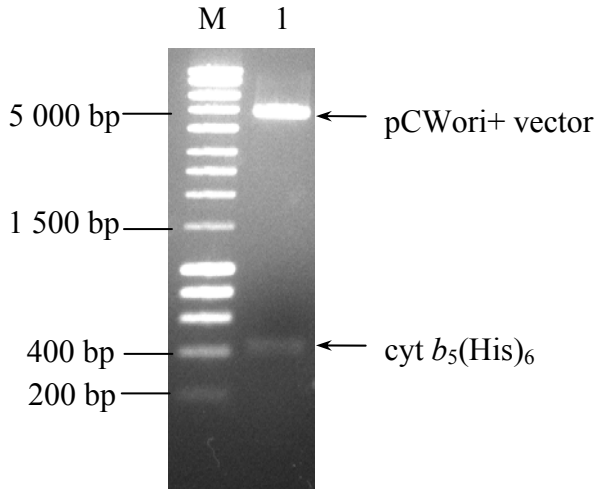


Figure 4.18: Construction of pC-cytb_5(His)$_6$. Double digest of pC-cytb_5(His)$_6$ using *Nde*I and *Hind*III restriction enzymes to confirm cytb_5(His)$_6$ insert. DNA was run on a 1% TAE agarose gel and viewed under UV light.

Expression of cytb_5(His)$_6$ was achieved in *E. coli*. A peak at about 420-430 nm was observed when reduced with sodium hydrosulphite (Figure 4.19). This peak is characteristics of cytb_5 expression.

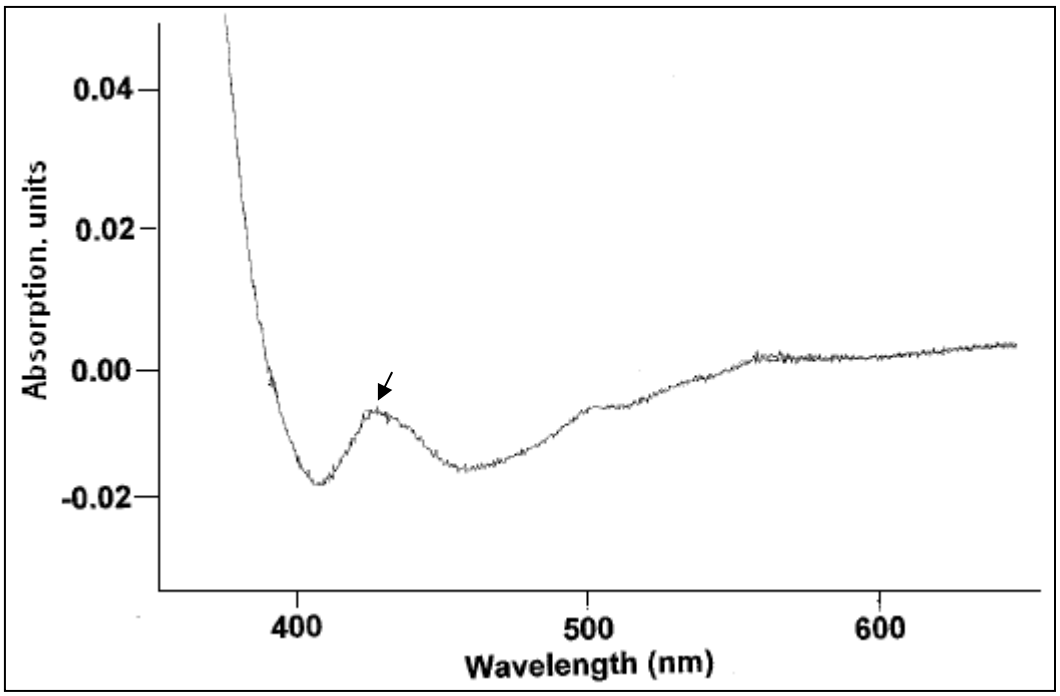


Figure 4.19: Absorbance of reduced CO - difference P450 spectrum. Cytb_5 expression is detected at an absorbance of about 420 nm.

4.5 Discussion

CYP6P9 is the first *An. funestus* protein to be expressed. No other protein studies in this species has ever been undertaken. The insect expression system developed by Qiagen was used to express *An. funestus* CYP6P9 in *E. coli* cells XL1 blue and BL21 (DE3) (pLysS). pIX4.0 was used as an expression vector in *E. coli,* due to the presence of the T7 promotor that is recognized by T7 RNA polymerase encoded in some *E. coli* (for instance *E. coli* BL21 strain). Protein expression under T7 promotor is achieved in the presence of inducer IPTG. Expression of CYP6P9 was unsuccessful even after the hydrophobic NH_2-terminus was truncated. Truncation of the hydrophobic region was done to improve expression. Expression of hydrophobic amino acids in *E. coli* is extremely difficult resulting in low or no expression. Attaching a His tag was performed in order to detect protein expression as well as for purification. No expression levels were detected on a Western blot and as a result, the expression vector was changed to a high regulated promotor (tac) expression plasmid, pCWori+. This vector has been used successfully in expressing other insect P450s in *E. coli* such as cockroach CYP15A1 (Helvig *et al.,* 2004).

Modifications of the NH_2- terminus is performed on the P450 sequence to enhance expression levels in *E. coli*. This system has been shown to express P450s in *E. coli* (Barnes *et al*., 1991). In this current work we employed the *ompA* (outer membrane protein A) leader sequence, a derivative of a bactieral membrane protein (Pritchard *et al.,* 1997). This bacterial signal peptide is fused to the NH_2- terminus of a P450 and

assists in the expression of hydrophobic NH_2- terminus P450s. Cleavage of the *ompA* is important for the expression of recombinant proteins. The addition of alanine (Ala) and proline (Pro) between the *ompA* and P450 gene allows for bacterial proteases to cleave at this site. The leader sequence *ompA* improves expression but reduces the catalytic activity of the P450 (Pritchard *et al.,* 2006). Pritchard *et al* (1997) used this method on human CYP3A4 and found it to have a much higher expression level compared to other techniques that use the 17α hydroxylase NH_2- terminal modification. This method, however, was not successful in expressing CYP6P9 in *E. coli.* A very low expression was noted for non-His tagged FUMOZ-R CYP6P9 when using the pCW-*ompA*2 vector. This vector has a Ala and Gly instead of the Ala/Pro. It is not clear whether this site is cleaved by bacterial signal peptidase. Growth conditions were optimized but with no success. Heme precursor (ALA) was added before cell inoculation so as to form heme molecules before induction to assist in P450 formation. This did not result in expression of CYP6P9. Sequcence analysis revealed that the *ompA* and P450 cDNA were in frame. This was important because it allowed translation to occur from the start codon (ATG) of *ompA* right through to the stop codon of CYP6P9 (TAG). A mismatch will result in early translational termination and hence no expression. Codon usage analysis reavealed only 18 out of 509 codons were rare in *E. coli.* This could result in low protein expression.

The *ompA* technique was successful in expressing CYP9J28 (*Aedes aegypti*) and *An. gambiae's* CYP6M2 and CYP6Z2 (Stevenson *et al.,* Unpublished data). As a result of unsuccessful expression of CYP6P9, the 17α hydroxylase method which was first

used in *E. coli* to produce bovine CYP17A by Barnes *et al* (1991) was employed. The first 8-18 amino acids depending on similarities are replaced with the 17α hydroxylase sequence (MALLLAVF) which is favoured by *E. coli* for protein expression. A second codon Ala (GCT) was used as it is the preferred codon by *E. coli* for the expression of proteins (Guzov *et al.,* 1996). Replacement of the first eight amino acids of CYP6P9 with 17α hydroxylase sequence produced high expression levels in *E. coli.* The first 24 hours after induction with IPTG and ALA produced no expression. The first expression was noted after 48 hours with the highest expression after 3 days. Expression after 48 hours may be due to the N-terminal modification (MALLLAVF) on CYP6P9, this was in contrast to the *ompA* sequence used in *An. gambiae's* CYP6M2 and CYP6Z2. Optimization of the N-terminal codons of P450 were shown to vary in P450 expression between 24-48 hours with varying P450 expression levels (Wu *et al.,* 2006a; Wu *et al.,* 2006b; Stark *et al.,* 2008). Saturation of carbon monoxide to CYP6P9 was achieved after 25 minutes. This however was in contrast to *An. gambiae's* CYP6M2 and CYP6Z2 which are saturated after three minutes (Stevenson *et al.,* Unpublished data).

Expression of cytb_5 was not problematic, this is due to the fact that the hydrophobic region is at the C-terminus rather than the NH_2-terminus. No expression was noted using the Qiagen pIX4.0 insect expression vector. Expression was observed when the pCWori+ expression vector was used.

Expression of *An. funestus* CPR was not attemped due to its lengthly preparation which involves insertion of a *pelB* leader sequence and subcloning into pCWori+ then into pACYC184 vectors. *Anopheles gambiea* CPR construct that was previously proven to express in *E. coli* was available and were used to eliminate unnecasary cost and time delays .

Chapter Five

General discussion and conclusions

5.1 Microsomal P450 activity (7-ethoxycoumarin assay)

Resistance to permethrin by FUMOZ-R was confirmed. This was shown by a zero percent knockdown within the first hour and 100% survival rate 24 hours post exposure. FANG however was susceptible to permethrin with 100% knockdown within the first hour and 100% mortality after 24 hours. Permethrin resistance in *An. funestus* FUMOZ-R was brought about by a metabolic based enzyme, cytochrome P450 (Brooke *et al.,* 2001). To further confirm that P450s were involved as a resistance mechanism, a P450 synergist (piperonyl butoxide (PBO)) was used that inhibits P450 activity (Kasai *et al*., 1999; Young *et al.,* 2005). Exposure to PBO inhibited P450s in FUMOZ-R as shown by a 93% knockdown rate within the first hour of permetrhin exposure and 100% mortality after 24 hours.

To determine the levels of P450 activity in both FUMOZ-R and FANG, microsomal P450 was isolated. Microsomes were isolated from adults as well as larval midguts. Heads from both adults and larvae were removed so as to prevent P450 inhibitor 'xanthommatin' (found within eye pigment) from coming into contact with microsomal extract (Danielson and Fogleman, 1994). Three day old, non blood fed adults were selected. Larval midguts were isolated and food content removed (food also inhibits P450 activity). In both strains larval midgut P450 had a higher activity

than those of headless adults. Kasai *et al* (2000) also observed that guts contained the highest P450 activity than any other tissues. However in *Drosophila,* P450 activity in adults was 20 times higher than in larvae (Danielson *et al*., 1994). The highest P450 activity was noted in FUMOZ-R larval midguts (4.127 ± 1.02 nmol/min/mg P450 protein). This was 3.6 times higher than that of FANG larval midguts (1.134 ± 0.58 nmol/min/mg P450 protein). FUMOZ-R headless adult P450 activity (1.573 ± 0.58 nmol/min/mg P450 protein) was 6.4 times higher than FANG headless adults (0.247 ± 0.09 nmol/min/mg P450 protein). The fold increase found in adult P450 from *An. funestus* was relatively higher compared with *An. gambiae* (2 fold increase) (Etang *et al.,* 2007)*, Culex quinquefasciatus* (1.6 and 2.5 fold increases for female and male adults respectively) (Chandre *et al.,* 1998) and *Aedes aegypti* (4 fold increase) (Prapanthadara *et al.,* 2002) but lower than that in the German cockroach (23 fold increase) (Siegfried and Scott 1992). The data presented here support the results of Brooke *et al* (2001) and go further in quantifying the fold expression.

5.2 HPLC analysis

Detoxification of permethrin by *An. funestus* microsomal P450 was further confirmed using an HPLC to detect the presence of metabolites produced. Two metabolites were detected at retention times 5.5 and 6 minutes from FUMOZ-R (headless adults) when incubated with 0.05 mM permethrin. Detoxification of permethrin produces two metabolites, 3-phenoxybenzyl alcohol (retention time 3.7 minutes) and 3-phenoxybenzoic acid (retention time 5.9 minutes) (Vontas *et al.,* 2001; Choi *et al.,*

2002). No metabolites were detected from FANG (headless adults). Extraction of permethrin from exposed mosquitoes (1-hour exposure and 20% knockdown) was not achieved due to the low sensitivity of the HPLC used. A gas chromatography-mass spectrometry (GC-MS) which is sensitive to small amounts of compounds is required to detect the presence of metabolites from these samples. Other instruments that may be used are electrospray mass spectrometry (ESMS) and liquid chromatography-electrospray mass spectrometry (LC-ESMS). Due to the unavailability these instruments were not used.

5.3 Analysis of *Anopheles funestus* CYP6P9

Amenya *et al* (2005) isolated the first partial P450 genes from *An. funestus.* One particular gene, CYP6P9 showed high levels of mRNA expression in FUMOZ-R (Amenya *et al*., 2008). Preliminary microarray analysis performed on FUMOZ-R and FANG showed an over-expression of CYP6P9 in FUMOZ-R (Naguran *et al.* unpublished data). Chromosomal mapping of a quantitative trait locus (QTL) associated with pyrethroid resistance in *An. funestus* (FUMOZ-R and FANG) was found to account for more than 60% of variance in susceptibility to permethrin. This QTL was situated between division 9 and 12 of chromosome arm 2R. This location however was seen to coincide with a cluster of CYP6 P450s which were mapped by fluorescent *in situ* hybridization. These results suggested that resistance was mediated by one or more CYP6 genes. CYP6P9 was found within this region (Wondji *et al.,* 2007).

From the above results, CYP6P9 was obviously a major candidate for pyrethroid resistance. Isolation and characterization of the full length CYP6P9 was needed to fully understand the mode of action. Full length CYP6P9 genes were isolated using specific primers designed using *An. gambiae* CYP6P3. CYP6P3 was shown to have an identity of 86% with that of CYP6P9 (Amenya *et al.,* 2005). An open reading frame cDNA sequence of CYP6P9 was isolated from both FUMOZ-R and FANG. Genomic DNA was also isolated. CYP6P9 amino acid sequence from FUMOZ-R and FANG shared a 99% identity with only four amino acid differences. Genomic DNA had only one intron that was of 56 bp in length. Multiple sequence alignment of CYP6P9 with other insect CYP6 genes showed a low percentage identity (≤ 45%). CYP6P9 contained all the characteristic features of a P450 which include: the heme-binding region (PFxxGxxxCxG), ExxR motif, ETLR domain and oxygen binding pocket [(A/G)Gx(E/D)T(T/S)] (characteristics of a cytochrome P450 protein). The presence of the YPDP motif indicated that it belonged to the CYP6 family (this motif is found only in CYP6 family). CYP6P9 is a membrane bound P450 as noted by the hydrophobic N-terminal region. The presence of the PERF motif also indicated that it was a microsomal P450. This motif is found in all microsomal cytochrome P450s. The major differences between FUMOZ-R and FANG CYP6P9 were noted in the F-G loop. Amino acid, Ile^{361} was replaced with an aromatic Phe^{361}. In other P450s the F-G loop operates as a lid, opening and closing to allow the entry and exit of substrates to the active site (Scott *et al.,* 2003; Poulos, 2003). The changes found in FUMOZ-R and FANG CYP6P9 may result in the efficiency of the enzyme to bind and detoxify permethrin. In *Drosophila melanogaster* three mutational changes

found in CYP6A2 gene from a resistant strain were shown to cause DDT resistance when compared with a susceptible strain (Bergé *et al.,* 1998; Amichot *et al.,* 2004). In order to determine the significance of the four changes noted in FUMOZ-R compared to FANG, site-directed mutagenesis is required in these regions to determine whether they cause resistance in FUMOZ-R.

While isolating CYP6P9 from FANG a novel CYP6 gene (CYP6P13) similar to that of CYP6P9 was isolated. This gene was mistaken for CYP6P9 but sequence alignment with FANG-CYP6P9 (from cDNA) showed a 93.7% identity. Phylogenetic analysis revealed that FUMOZ-R and FANG CYP6P9s were closely related while FANG CYP6P13 was on a different branch indicating that it was a different gene. Amino acid sequence alignment revealed a total of 32 differences. The novel gene was given the name CYP6P13 by the P450 Nomenclature Committee (http://drnelson.utmem.edu/CytochromeP450.html). CYP6P13 also had one intron which was 57 bp, this was 1 bp longer than CYP6P9. CYP6P13 was only found in FANG genomic DNA. The two strains FUMOZ-R and FANG were originally from two different geological locations (FUMOZ-R from Mozambique and FANG from Angola) and this could account for the differences seen (e.g. CYP6P13 could be a duplication in FANG, but is not expressed).

5.4 Analysis of *Anopheles funestus* Cytb_5 and CPR

Anopheles funestus redox partners, cytochrome b_5 and NADPH-cytochrome P450 reductase were also isolated. *Anopheles funestus* cytb_5 was shown to have a 97% identity with that of *An. gambiae* cytb_5. Multiple sequence alignment with other insect P450s showed that *An. funestus* cytb_5 contained all the features of a cytb_5 protein which are the "b_5 fold" and two histidines that are required for heme-coordination (Guzov *et al*., 1996; Nikou *et al*., 2003). A hydrophobic C-terminal was noted which acts as a membrane anchor.

Anopheles funestus CPR was also isolated and it was found to have a 96.5% identity with that of *An. gambiae* CPR. It had all the characteristic features for CPR which includes: the FMN and FAD domains, the conserved NADPH binding residues as well as the two tyrosine residues required for the binding of FMN. Electrons are catalyzed within the FAD/$NADP^+$ domains of CPR and then transferred to cytochrome P450s via the FMN domain (Smith *et al.,* 1994; Wang *et al.,* 1997). The first of the two electrons donated to P450 by redox partners is from CPR (Vergères and Waskell, 1995). Electrons are transferred to cytochrome P450 via the FMN domain that has been shown to bind to the proximal surface of cytochrome P450 (Sevrioukova *et al*., 1999a; Sevrioukova *et al*., 1999b). The presence of a hydrophobic N-terminal indicates that CPR is membrane bound.

5.5 *Escherichia coli* expression of CYP6P9

This current work is the first to report on the expression of CYP6P9 protein. No *An. funestus* proteins have ever been expressed before. The insect expression vector pIX 4.0 developed by Qiagen was used for the expression of CYP6P9. Expression was performed in *E. coli* cells XL1 Blue and BL21 (DE3)(pLSs). However, expression was unsuccessful even after the hydrophobic NH_2-terminus was truncated. A six His tag was attached to detect the expression of proteins when analyzed using the Western blot analysis. No protein fragments were detected on the Western blot indicating that expression was not achieved. Expression vector pIX 4.0 was not a suitable expression system for the expression of P450 proteins. Many or all P450s are succesfully expressed using a high regulated tac promotor expression plasmid, pCWori+. Eukaryotes and prokaryotes P450 proteins have been expressed using this plasmid (Barnes *et al*., 1991; Helvig *et al.,* 2004).

Expression of membrane P450 proteins is only achieved by modification of the NH_2-terminus (Barnes *et al*., 1991). This involves replacing the first amino acids with that of 17α hydroxylase sequence (MALLLAVF). Pritchard *et al* (1997) developed a technique that involved fusing a bacterial leader sequence at the NH_2-terminus of the P450 gene. The bacterial leader sequence aided in expressing P450 proteins in *E. coli* without the need of modifying the NH_2-terminus. Bacterial leader sequences used are the *ompA* (outer membrane protein A) and *pelB* (pectate lyase B). It was important to add two amino acids Ala and Pro between the bacterial leader sequence and P450 gene so as to allow bacterial proteases to cleave the leader sequence from P450

during expression. Human CYP3A4 has been successfully expressed in *E. coli* using this technique (Pritchard *et al*., 1997). CYP9J28 in *Aedes aegypti* and CYP6M2 and CYP6Z2 in *An. gambaie* have recently been expressed in *E. coli* under the control of '*ompA'* (Stevenson *et al.,* Unpublished data).

The bacterial leader sequence '*ompA'* was attached to CYP6P9 and expressed in *E. coli* DH5α cells. No expression was observed. Redox partners cytb_5 and CPR were co-expressed with CYP6P9 to assist in expression but still no expression was observed. Low levels of expressions were noted in pCW-*ompA*2CYP6P9. This plasmid was constructed by attaching *ompA* leader sequence and introducing a *NgoM*VI restriction site (Ala and Gly) adjacent to the leader sequence to allow cloning of P450 genes. A second construct was produced which had a six His tag attached to the C-terminal and fusing the *ompA* leader sequence to the NH_2 terminal of CYP6P9 before ligating into pCWori+ plasmid. No expression was detected using this construct. Western blot analysis detected no protein fragments indicating that no proteins were expressed. *Escherichia coli* expression was optimized by varying growth conditions. This included temperature change (25-30 ºC), addition of ALA before cell inoculation to allow for the formation of heme molecule to increase the chases of CYP6P9 expression and allowing induction to occur for three days. No expression was achieved. Sequence analysis indicated that the *ompA* sequence was in frame and that the gene contained no insertions or deletions that may result in translational termination. Rare codon usage in *E. coli* was checked and only 3.5% of the total gene was rare to *E. coli* usage. This indicated that rare codon usage was not

the major reason for the lack of expression. Due to the failure of expression using the bacterial leader sequence (*ompA*), expression was changed to the 17 α hydroxylase technique. No expression was obtained after the first 24 hours post induction with IPTG and ALA. Expression of 17α-CYP6P9 was finally noted after two days and the highest expression on day three. Carbon monoxide stauration was achieved after 25 minutes. This however was in contrast to *An. gambiae's* CYP6M2 and CYP6Z2 which are saturated after three minutes (Stevenson *et al.,* Unpublished data). These results showed that expression of *An. funestus* CYP6P9 in *E. coli* was problematic. Bacterial leader sequence (*ompA*) did not impove the expression of CYP6P9. Expression of CYP6P9 was only achieved using the 17 α hydroxylase technique, however induction was slow. This was noted with a low expression on day two and high expression on day three post induction with IPTG and ALA. P450 induction is usually one to two days long (Pritchard *et al.,* 2006).

Based on results above it seems that CYP6P9 might have a higher affinity for its substrate (permethrin) therefore resulting in better detoxification of permethrin. The fact that this same gene is also overexpressed and indirectly implicating increase enzyme levels might be significant and shoud be further investigated. Having both types of gene alterations will make this an extremely effective system. However, such a system has never been reported.

Expression of cytb_5 was not achieved using insect expression vector pIX4.0 but was achieved using pCWori+ expression vector. Unlike CYP6P9, expression was not problematic. An expression peak was observed at around 420 nm. This peak is characteristic of cytb_5 expression. House fly cytb_5 was shown to have three characteristic peaks, A large peak at 420nm and two small peaks at 527 and 557 nm (Guzov *et al.,* 1996). *Anopheles funestus* CPR did not express using insect expression vector pIX4.0. CPR is usually expressed in the pACYC184 plasmid but due to time constraints it was not constucted. *Anopheles gambiae* CPR (pACYC-*pelB*CPR) was available and was used in co-expression with CYP6P9. *Anopheles gambiae* and *An. funestus* CPRs share a 96.5 % identity.

5.6 Future work

Further investigation is required to extract metabolites formed as a result of permethrin detoxification in exposed FUMOZ-R. Detection can be performed using a gas chromatography-mass spectrometry (GC-MS) which is sensitive to small amounts of compounds. These finding will determine the compounds formed during permethrin detoxification by FUMOZ-R. Enzyme activity of FUMOZ-R and FANG CYP6P9 are required to compare the levels of activities as well as enzyme kinetics such as V_{max}, K_m and k_{cat}. Site directed mutagenesis on the four amino acid differences noted in FUMOZ-R CYP6P9 and FANG CYP6P9 is required to determine their effects in CYP6P9 enzyme activity. These results will indicate which amino acid is important during enzyme detoxification of permethrin. Antibodies

targeted against CYP6P9 proteins can be produced and used to compare and determine elevated expression levels of CYP6P9 protein from both FANG and FUMOZ-R. Gene knockdown using RNAi technique could be employed. This technique is used to target genes of interest (CYP6P9) by inhibiting gene expression at the translation stage or blocking the transcription of specific genes. In the case of CYP6P9, this will result in the pyrethroid resistant strain, FUMOZ-R being susceptible which will indicate that CYP6P9 plays a major role in pyrethroid resistance. These types of studies have been done in *Anopheles gambiae* P450 reducase (Lycett *et al*., 2006).

In conclusion, this study has advanced our knowledge of resistance mechanism in *An. funestsus* by understanding the role of cytochrome P450s in the detoxification of permethrin. Work carried out by Amenya *et al* (2005 and 2008) provided evidence that CYP6P9 was the major candidate associated with insecticide resistance. Mutational changes found between CYP6P9 from resistant and susceptible strains may account for the differences in enzyme activity. However, this needs to be investigated further. Understanding of the mechanism of P450 enzyme detoxification will contribute towards the understanding of insecticide resistance and ultimately in the development of novel improved methods of vector control.

Appendix

A1: Recipe for media preparations

1. Luria Bertani Broth (1 litre)

   10g Bacto-tryptone
   5g Bacto-Yeast extract
   10g NaCl
   Adjust to 1L with distilled water and autoclave

2. Luria Bertani Agar (1 litre)

   10g Bacto-tryptone
   5g Bacto-Yeast extract
   10g NaCl
   15g Bacto-Agar
   Adjust to 1L with distilled water and autoclave

   Pour to plates when cooled.

3. SOC Media (1 Litre)

   20g Bacto Tryptone,
   5g, Bacto Yeast Extract
   2ml 5M NaCl
   2.5ml 1M KCl
   10ml 1M $MgCl_2$
   10ml 1M $MgSO_4$
   20ml 1M glucose
   Adjust to 1L with distilled water and autoclave.

4. Terrific Broth (1 Litre)

   12g bacto-tryptone;
   24g bacto-yeast extract,
   2g bacto-peptone;
   4ml glycerol in
   Adjust to 900ml with distilled water

   Add 100 ml of a sterile 10x phosphate solution before use

   10x phosphate solution (0.17 M KH_2PO_4 and 0.72 M K_2HPO_4)

A2: Protein standard curve

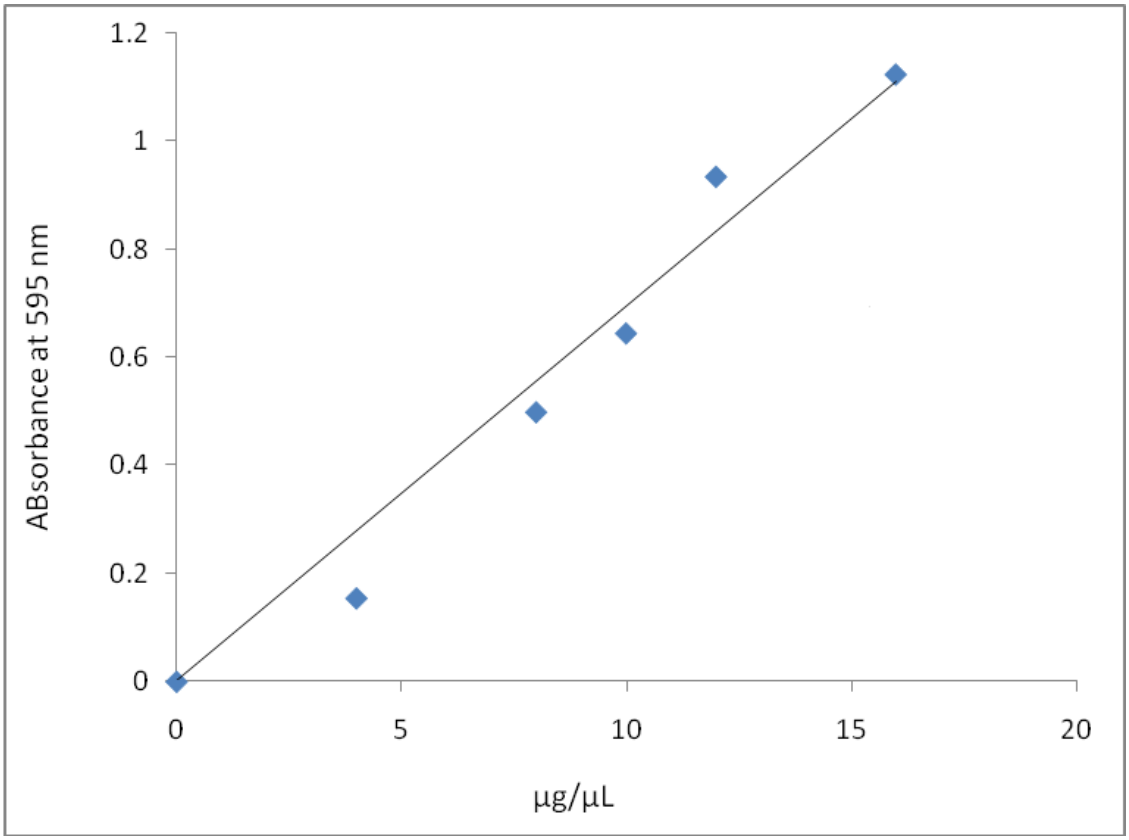


Figure A2.1: Protein standard curve. Bradford's reagent was use to detemine the concentration of P450 Protein. The curve had an R^2 value of 0.966 and the equation used was y= 0.069x.

A3: 7- hydroxycoumarin Standard curve

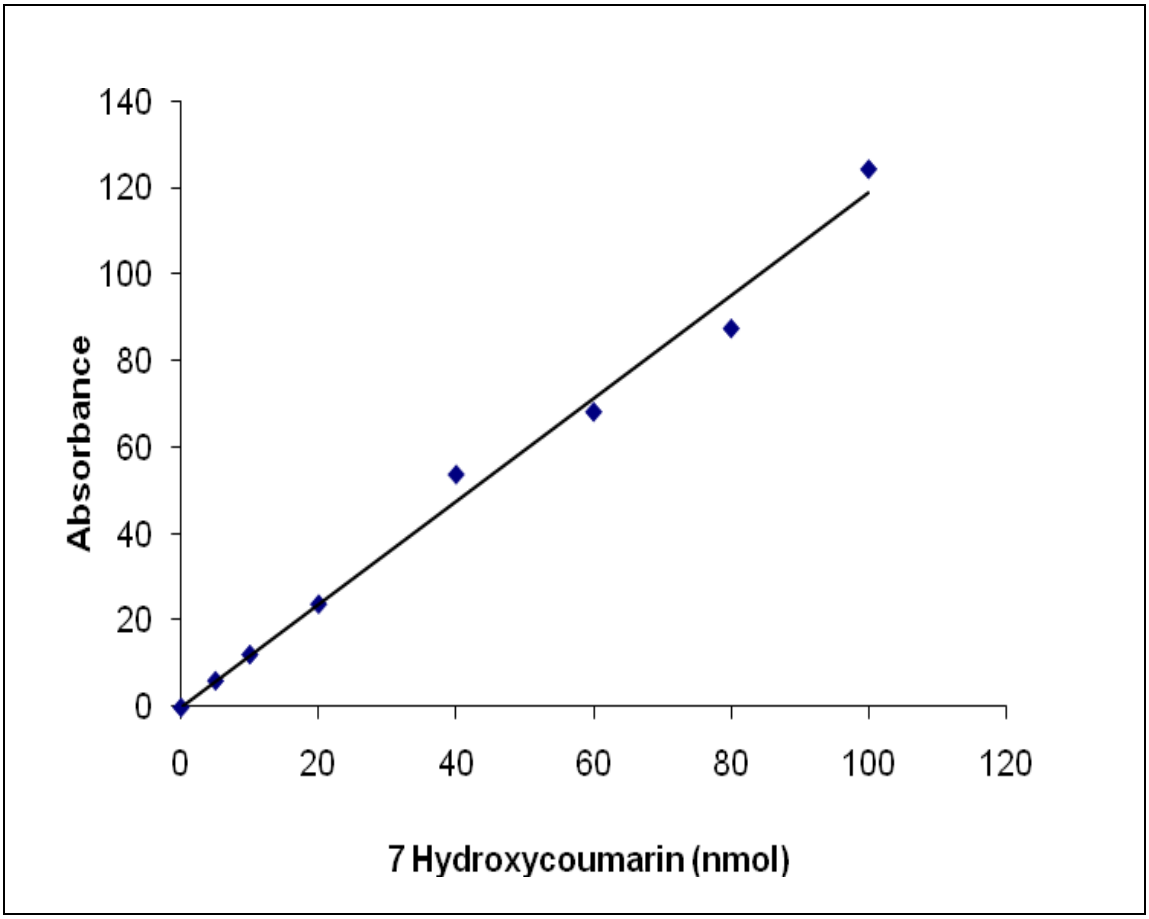


Figure A3.1: The 7-hydroxycoumarin standard curve. The 7-hydroxycoumarin standard curve was used to detemine the concentration of 7-hydroxycoumarin formed from the hydrolysis of 7-ethoxycoumarin by microsomal P450 Proteins. The curve had an R^2 value of 0.990 and the equation used was y= 1.188x.

A4: Nucleotide and Amino acid sequences of CYP6P9

CTCGAGCATCATCACCATCACCACATGGCTGGACACGCGAAAGGACAGGCCCAGACAAGGCATGCGGC
CGACATCCATCTGGAACTGTACAAAAAATTCAAGCAGCGCCGTGAGCGGTACGTTGGTATGAGCCAGT
TCATGATACCTTCATTGCTCGTGATCGATCCAGAGCTGGTGAAAACGATCCTAGTAAAGGACTTTAAT
GTATTCCACGATCATGGTGTATTCAATAATGCAAGAGACGACCCGCTGTCCGCACATCTTTTTGCGCT
TGAAGGTAACCCATGGCGCTTGTTGCGTCAGAAGCTCACGCCAACGTTCACCTCAGGTCGCATGAAGC
AAATGTTTGGTACACTATGGGATGTAGCACTTGAGCTGGACAAGTATATGGAAGAAAACTATCGTCAG
CCGGATATTGAGATGAAGGATGTGCTAGGTCGGTTTACGACAGATGTGATTGGCACCTGTGCATTCGG
GATTGAGTGTAATACGCTTAAGACACCGGACTCGGAGTTCCGCAAATACGGCAACAAAGCGTTTGAGT
TTAATCTGATGATTTTTCTAAAAACTTTCTTAGCATCGGCTTATCCGTCACTTGTGCGGAAACTGCGA
ATGAAGATAACATTCGACGATGTGGAACAGTTTTTCCTAAAAATTGTTAAGGAAACGGTGGAATATCG
AGAAAGTAACAACATTAAACGAAACGACTTCATGAACCTGCTGTTGCAGATTAAGAATAAGGGTAAGC
TGGACGACAGCGATGATGGGAGTGTTGGCAAGGGTGAAGTAGGAATGACACAACGAGAACTTGCGGCA
CAGGCATTCATTTTCTTCTTGGCCGGTTTCGAGACATCATCCACGACGCAAAGCTTCTGTCTGTACGA
GTTGGCAAAGAACCCTGACATCCAGGAGCGCCTTAGACAAGAGATCAACCAAGCAATCGAGGAGAATG
ACGGCCAGGTGACGTACGATGTCGCCATGAACATACAGTATCTGGACAATGTGATAAACGAAACACTT
CGCAAGTACCCACCGGTAGAATCGTTGAGTCGTGTGCCGTCTGTTGACTATGTCATCCCAGGTACGAA
ACATGTCATTCCCAAACGAACGTTAGTGCAAATTCCGGTTCACGCCATTCAACATGATCCTGAGCACT
ATCCCGATCCAGAACGTTTCGATCCGGATCGCTTCTCACCGGAGGAAGTGAAGAAGCGACATCCCTTC
ACGTTCCTCCCATTCGGTGAGGGGCCACGCGTTTGCATTGGGCTTCGGTTTGGTGTGATGCAGACGAA
GGTAGGATTGATAACGCTGTTGAGAAAGTTCCGCTTCTCACCGTCAGCGCGTACACCAGATTGTGTAA
AGTTCGATCCGAAAATGATCATTCTGTCACCGATCGCGGGTAATTACTTGAAGGTGGAAAAGTTGTAG
TCTAGA

L E **H H H H H H** **Met** A G H A K G Q A Q T R H A A D I H L E L Y K K F K Q R R E R Y V G
Met S Q F **Met** I P S L L V I D P E L V K T I L V K D F N V F H D H G V F N N A R D D P L S A
H L F A L E G N P W R L L R Q K L T P T F T S G R **Met** K Q **Met** F G T L W D V A L E L D K Y
Met E E N Y R Q P D I E **Met** K D V L G R F T T D V I G T C A F G I E C N T L K T P D S E F R
K Y G N K A F E F N L **Met** I F L K T F L A S A Y P S L V R K L R **Met** K I T F D D V E Q F F L
K I V K E T V E Y R E S N N I K R N D F **Met** N L L L Q I K N K G K L D D S D D G S V G K G
E V G **Met** T Q R E L A A Q A F I F F L A G F E T S S T T Q S F C L Y E L A K N P D I Q E R L
R Q E I N Q A I E E N D G Q V T Y D V A **Met** N I Q Y L D N V I N E T L R K Y P P V E S L S R
V P S V D Y V I P G T K H V I P K R T L V Q I P V H A I Q H D P E H Y P D P E R F D P D R F S
P E E V K K R H P F T F L P F G E G P R V C I G L R F G V **Met** Q T K V G L I T L L R K F R F S
P S A R T P D C V K F D P K **Met** I I L S P I A G N Y L K V E K L **Stop** S R

Figure A4.1: Sequences of $(His)_6$CYP6P9. Restriction enzymes are underlined, *Xho*I (before start codon) and *Xba*I (After stop codon). A 6 x His-tag is in blue. Hydrophobic amino acids were removed. Alanine was added as a second codon

CATATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTAGCGCAGGCC**GC**
GCCGATGGAGCTAATTAACGCGGTGTTGGCCGCGTTCATCTTCGTAGTGTCGGCAGTGTACCTTTTCA
TTCGGAACAAACATAATTACTGGAAAGACAATGGATTCCCGTATGCGCCGAACCCACATTTTCTGTTC
GGACACGCGAAAGGACAGGCCCAGACAAGGCATGCGGCCGACATCCATCTGGAACTGTACAAAAAATT
CAAGCAGCGCCGTGAGCGGTACGTTGGTATGAGCCAGTTCATGATACCTTCATTGCTCGTGATCGATC
CAGAGCTGGTGAAAACGATCCTAGTAAAGGACTTTAATGTATTCCACGATCATGGTGTATTCAATAAT
GCAAGAGACGACCCGCTGTCCGCACATCTTTTTGCGCTTGAAGGTAACCCATGGCGCTTGTTGCGTCA
GAAGCTCACGCCAACGTTCACCTCAGGTCGCATGAAGCAAATGTTTGGTACACTATGGGATGTAGCAC
TTGAGCTGGACAAGTATATGGAAGAAAACTATCGTCAGCCGGATATTGAGATGAAGGATGTGCTAGGT
CGGTTTACGACAGATGTGATTGGCACCTGTGCATTCGGGATTGAGTGTAATACGCTTAAGACACCGGA
CTCGGAGTTCCGCAAATACGGCAACAAAGCGTTTGAGTTTAATCTGATGATTTTTCTAAAAACTTTCT
TAGCATCGGCTTATCCGTCACTTGTGCGGAAACTGCGAATGAAGATAACATTCGACGATGTGGAACAG
TTTTTCCTAAAAATTGTTAAGGAAACGGTGGAATATCGAGAAAGTAACAACATTAAACGAAACGACTT
CATGAACCTGCTGTTGCAGATTAAGAATAAGGGTAAGCTGGACGACAGCGATGATGGGAGTGTTGGCA
AGGGTGAAGTAGGAATGACACAACGAGAACTTGCGGCACAGGCATTCATTTTCTTCTTGGCCGGTTTC
GAGACATCATCCACGACGCAAAGCTTCTGTCTGTACGAGTTGGCAAAGAACCCTGACATCCAGGAGCG
CCTTAGACAAGAGATCAACCAAGCAATCGAGGAGAATGACGGCCAGGTGACGTACGATGTCGCCATGA
ACATACAGTATCTGGACAATGTGATAAACGAAACACTTCGCAAGTACCCACCGGTAGAATCGTTGAGT
CGTGTGCCGTCTGTTGACTATGTCATCCCAGGTACGAAACATGTCATTCCCAAACGAACGTTAGTGCA
AATTCCGGTTCACGCCATTCAACATGATCCTGAGCACTATCCCGATCCAGAACGTTTCGATCCGGATC
GCTTCTCACCGGAGGAAGTGAAGAAGCGACATCCCTTCACGTTCCTCCCATTCGGTGAGGGGCCACGC
GTTTGCATTGGGCTTCGGTTTGGTGTGATGCAGACGAAGGTAGGATTGATAACGCTGTTGAGAAAGTT
CCGCTTCTCACCGTCAGCGCGTACACCAGATTGTGTAAAGTTCGATCCGAAAATGATCATTCTGTCAC
CGATCGCGGGTAATTACTTGAAGGTGGAAAAGTTG**CATCATCACCATCACCAC**TAG**TCTAGA**

H **Met** K K T A I A I A V A L A G F A T V A Q A **A** **P** **Met** E L I N A V L A A F I F V V S A V Y
L F I R N K H N Y W K D N G F P Y A P N P H F L F G H A K G Q A Q T R H A A D I H L E L Y
K K F K Q R R E R Y V G **Met** S Q F **Met** I P S L L V I D P E L V K T I L V K D F N V F H D H G
V F N N A R D D P L S A H L F A L E G N P W R L L R Q K L T P T F T S G R **Met** K Q **Met** F G
T L W D V A L E L D K Y **Met** E E N Y R Q P D I E **Met** K D V L G R F T T D V I G T C A F G I
E C N T L K T P D S E F R K Y G N K A F E F N L **Met** I F L K T F L A S A Y P S L V R K L R
Met K I T F D D V E Q F F L K I V K E T V E Y R E S N N I K R N D F **Met** N L L L Q I K N K G
K L D D S D D G S V G K G E V G **Met** T Q R E L A A Q A F I F F L A G F E T S S T T Q S F C L
Y E L A K N P D I Q E R L R Q E I N Q A I E E N D G Q V T Y D V A **Met** N I Q Y L D N V I N E
T L R K Y P P V E S L S R V P S V D Y V I P G T K H V I P K R T L V Q I P V H A I Q H D P E H
Y P D P E R F D P D R F S P E E V K K R H P F T F L P F G E G P R V C I G L R F G V **Met** Q T
K V G L I T L L R K F R F S P S A R T P D C V K F D P K **Met** I I L S P I A G N Y L K V E K L H
H H H H H H **Stop** S R

Figure A4.2: Sequences of *ompA*-CYP6P9(His)$_6$. Restriction enzymes are underlined, *Nde*I (before start codon) and *Xba*I (After stop codon). Bacterial leader sequence (*ompA*) is highlighted in red. The two amino acids alanine and proline (site for bacterial proteases) are in green. A 6 x His-tag is in blue.

CATATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTAGCGCAGGCC**GC**
GCCGATGGAGCTAATTAACGCGGTGTTGGCCGCGTTCATCTTCGTAGTGTCGGCAGTGTACCTTTTCA
TTCGGAACAAACATAATTACTGGAAAGACAATGGATTCCCGTATGCGCCGAACCCACATTTTCTGTTC
GGACACGCGAAAGGACAGGCCCAGACAAGGCATGCGGCCGACATCCATCTGGAACTGTACAAAAAATT
CAAGCAGCGCCGTGAGCGGTACGTTGGTATGAGCCAGTTCATGATACCTTCATTGCTCGTGATCGATC
CAGAGCTGGTGAAAACGATCCTAGTAAAGGACTTTAATGTATTCCACGATCATGGTGTATTCAATAAT
GCAAGAGACGACCCGCTGTCCGCACATCTTTTTGCGCTTGAAGGTAACCCATGGCGCTTGTTGCGTCA
GAAGCTCACGCCAACGTTCACCTCAGGTCGCATGAAGCAAATGTTTGGTACACTATGGGATGTAGCAC
TTGAGCTGGACAAGTATATGGAAGAAAACTATCGTCAGCCGGATATTGAGATGAAGGATGTGCTAGGT
CGGTTTACGACAGATGTGATTGGCACCTGTGCATTCGGGATTGAGTGTAATACGCTTAAGACACCGGA
CTCGGAGTTCCGCAAATACGGCAACAAAGCGTTTGAGTTTAATCTGATGATTTTTCTAAAAACTTTCT
TAGCATCGGCTTATCCGTCACTTGTGCGGAAACTGCGAATGAAGATAACATTCGACGATGTGGAACAG
TTTTTCCTAAAAATTGTTAAGGAAACGGTGGAATATCGAGAAAGTAACAACATTAAACGAAACGACTT
CATGAACCTGCTGTTGCAGATTAAGAATAAGGGTAAGCTGGACGACAGCGATGATGGGAGTGTTGGCA
AGGGTGAAGTAGGAATGACACAACGAGAACTTGCGGCACAGGCATTCATTTTCTTCTTGGCCGGTTTC
GAGACATCATCCACGACGCAAAGCTTCTGTCTGTACGAGTTGGCAAAGAACCCTGACATCCAGGAGCG
CCTTAGACAAGAGATCAACCAAGCAATCGAGGAGAATGACGGCCAGGTGACGTACGATGTCGCCATGA
ACATACAGTATCTGGACAATGTGATAAACGAAACACTTCGCAAGTACCCACCGGTAGAATCGTTGAGT
CGTGTGCCGTCTGTTGACTATGTCATCCCAGGTACGAAACATGTCATTCCCAAACGAACGTTAGTGCA
AATTCCGGTTCACGCCATTCAACATGATCCTGAGCACTATCCCGATCCAGAACGTTTCGATCCGGATC
GCTTCTCACCGGAGGAAGTGAAGAAGCGACATCCCTTCACGTTCCTCCCATTCGGTGAGGGGCCACGC
GTTTGCATTGGGCTTCGGTTTGGTGTGATGCAGACGAAGGTAGGATTGATAACGCTGTTGAGAAAGTT
CCGCTTCTCACCGTCAGCGCGTACACCAGATTGTGTAAAGTTCGATCCGAAAATGATCATTCTGTCAC
CGATCGCGGGTAATTACTTGAAGGTGGAAAAGTTGTAG**TCTAGA**

H **Met** K K T A I A I A V A L A G F A T V A Q A **A P** **Met** E L I N A V L A A F I F V V S A V Y
L F I R N K H N Y W K D N G F P Y A P N P H F L F G H A K G Q A Q T R H A A D I H L E L Y
K K F K Q R R E R Y V G **Met** S Q F **Met** I P S L L V I D P E L V K T I L V K D F N V F H D H G
V F N N A R D D P L S A H L F A L E G N P W R L L R Q K L T P T F T S G R **Met** K Q **Met** F G
T L W D V A L E L D K Y **Met** E E N Y R Q P D I E **Met** K D V L G R F T T D V I G T C A F G I
E C N T L K T P D S E F R K Y G N K A F E F N L **Met** I F L K T F L A S A Y P S L V R K L R
Met K I T F D D V E Q F F L K I V K E T V E Y R E S N N I K R N D F **Met** N L L L Q I K N K G
K L D D S D D G S V G K G E V G **Met** T Q R E L A A Q A F I F F L A G F E T S S T T Q S F C L
Y E L A K N P D I Q E R L R Q E I N Q A I E E N D G Q V T Y D V A **Met** N I Q Y L D N V I N E
T L R K Y P P V E S L S R V P S V D Y V I P G T K H V I P K R T L V Q I P V H A I Q H D P E H
Y P D P E R F D P D R F S P E E V K K R H P F T F L P F G E G P R V C I G L R F G V **Met** Q T
K V G L I T L L R K F R F S P S A R T P D C V K F D P K **Met** I I L S P I A G N Y L K V E K L
Stop S R

Figure A4.3: Sequences of *ompA*-CYP6P9. Restriction enzymes are underlined, *Nde*I (before start codon) and *Xba*I (After stop codon). Bacterial leader sequence (*ompA*) is highlighted in red. The two amino acids alanine and proline (site for bacterial proteases) are in green.

CATATGGCTCTGTTATTAGCAGTTTTTGCCGCGTTCATCTTCGTAGTGTCGGCAGTGTACCTTTTCAT
TCGGAACAAACATAATTACTGGAAAGACAATGGATTCCCGTATGCGCCGAACCCACATTTTCTGTTCG
GACACGCGAAAGGACAGGCCCAGACAAGGCATGCGGCCGACATCCATCTGGAACTGTACAAAAAATTC
AAGCAGCGCCGTGAGCGGTACGTTGGTATGAGCCAGTTCATGATACCTTCATTGCTCGTGATCGATCC
AGAGCTGGTGAAAACGATCCTAGTAAAGGACTTTAATGTATTCCACGATCATGGTGTATTCAATAATG
CAAGAGACGACCCGCTGTCCGCACATCTTTTTGCGCTTGAAGGTAACCCATGGCGCTTGTTGCGTCAG
AAGCTCACGCCAACGTTCACCTCAGGTCGCATGAAGCAAATGTTTGGTACACTATGGGATGTAGCACT
TGAGCTGGACAAGTATATGGAAGAAAACTATCGTCAGCCGGATATTGAGATGAAGGATGTGCTAGGTC
GGTTTACGACAGATGTGATTGGCACCTGTGCATTCGGGATTGAGTGTAATACGCTTAAGACACCGGAC
TCGGAATTCCGCAAATACGGCAACAAAGCGTTTGAGTTTAATCTGATGATTATTCTAAAAACTTTCTT
AGCATCGGCTTATCCGTCACTTGTGCGGAAACTGCGAATGAAGATAACATTCGACGATGTGGAACAGT
TTTTCCTAAAAATTGTTAAGGAAACGGTGGAATATCGAGAAAGTAACAACATTAAACGAAACGACTTC
ATGAACCTGCTGTTGCAGATTAAGAATAAGGGTAAGCTGGACGACAGCGATGATGGGAGTGTTGGCAA
GGGTGAAGTAGGAATGACACAACGAGAACTTGCGGCACAGGCATTCATTTTCTTCTTGGCCGGTTTCG
AGACATCATCCACGACGCAAAGCTTCTGTCTGTACGAGTTGGCAAAGAACCCTGACATCCAGGAGCGC
CTTAGACAAGAGATCAACCAAGCAATCGAGGAGAATGACGGCCAGGTGACGTACGATGTCGCCATGAA
CATACAGTATCTGGACAATGTGATAAACGAAACACTTCGCAAGTACCCACCGGTAGAATCGTTGAGTC
GTGTGCCGTCTGTTGACTATGTCATCCCAGGTACGAAACATGTCATTCCCAAACGAACGTTAGTGCAA
ATTCCGGTTCACGCCATTCAACATGATCCTGAGCACTATCCCGATCCAGAACGTTTCGATCCGGATCG
CTTCTCACCGGAGGAAGTGAAGAAGCGACATCCCTTCACGTTCCTCCCATTCGGTGAGGGGCCACGCG
TTTGCATTGGGCTTCGGTTTGGTGTGATGCAGACGAAGGTAGGATTGATAACGCTGTTGAGAAAGTTC
CGCTTCTCACCGTCAGCGCGTACACCAGATTGTGTAAAGTTCGATCCGAAAATGATCATTCTGTCACC
GATCGCGGGTAATTACTTGAAGGTGGAAAAGTTGTAG**TCTAGA**

H M A L L L A V F A A F I F V V S A V Y L F I R N K H N Y W K D N G F P Y A P N P H F L F
G H A K G Q A Q T R H A A D I H L E L Y K K F K Q R R E R Y V G M S Q F M I P S L L V I D
P E L V K T I L V K D F N V F H D H G V F N N A R D D P L S A H L F A L E G N P W R L L R
Q K L T P T F T S G R M K Q M F G T L W D V A L E L D K Y M E E N Y R Q P D I E M K D V
L G R F T T D V I G T C A F G I E C N T L K T P D S E F R K Y G N K A F E F N L M I I L K T F
L A S A Y P S L V R K L R M K I T F D D V E Q F F L K I V K E T V E Y R E S N N I K R N D F
M N L L L Q I K N K G K L D D S D D G S V G K G E V G M T Q R E L A A Q A F I F F L A G F
E T S S T T Q S F C L Y E L A K N P D I Q E R L R Q E I N Q A I E E N D G Q V T Y D V A Met
N I Q Y L D N V I N E T L R K Y P P V E S L S R V P S V D Y V I P G T K H V I P K R T L V Q I
P V H A I Q H D P E H Y P D P E R F D P D R F S P E E V K K R H P F T F L P F G E G P R V C I
G L R F G V Met Q T K V G L I T L L R K F R F S P S A R T P D C V K F D P K M I I L S P I A G
N Y L K V E K L Stop S R

Figure A4.4: Sequences of 17α-CYP6P9. Restriction enzymes are underlined, *Nde*I (before start codon) and *Xba*I (After stop codon). The 17 α hydroxylase nucleotide and amino acid sequence are indicated in red.

A5: Nucleotide and Amino acid sequences of cytb_5

CTCGAGCATCATCACCATCACCACATGGCTGAAGTGAAAACGTATTCGCTGGCGGAGGTGAAATCCCA
CAACACCAACAAATCGACCTGGATCGTAATTCACAACGATATCTTCGACGTGACGGAGTTTCTGAACG
AGCATCCTGGCGGAGAAGAAGTGCTGCTAGAGCAGGCCGGTAAGGAAGCAACGGAAGCATTCGAGGAT
GTGGGCCACAGCAGCGATGCCCGCGAGATGATGAAGAAGTTTAAAGTGGGCGAACTGATCGAATCCGG
ACGCAAACAGGTCCCGGTAAAGAAGGAACCGGATTGGAAATCGGAGCAGCAGGATGATAATCAACTAA
AGCAATGGATAGTGCCACTTATCCTGGGTCTGCTCGCAACCATCCTCTATCGGTTCTACTTCACCCAG
TAA**CTGCAG**

L E **H H H H H H** **Met** A E V K T Y S L A E V K S H N T N K S T W I V I H N D I F D V T E F L
N E H P G G E E V L L E Q A G K E A T E A F E D V G H S S D A R E **Met** **Met** K K F K V G E L
I E S G R K Q V P V K K E P D W K S E Q Q D D N Q L K Q W I V P L I L G L L A T I L Y R F Y
F T Q **Stop** L Q

Figure A5.1: Sequences of (His)$_6$cytb_5. Restriction enzymes are underlined, *Xho*I (before start codon) and *Pst*I (After stop codon). Six His –tag is indicated in blue.

CATATGGCTGAAGTGAAAACGTATTCGCTGGCGGAGGTGAAATCCCACAACACCAACAAATCGACCTG
GATCGTAATTCACAACGATATCTTCGACGTGACGGAGTTTCTGAACGAGCATCCTGGCGGAGAAGAAG
TGCTGCTAGAGCAGGCCGGTAAGGAAGCAACGGAAGCATTCGAGGATGTGGGCCACAGCAGCGATGCC
CGCGAGATGATGAAGAAGTTTAAAGTGGGCGAACTGATCGAATCCGGACGCAAACAGGTCCCGGTAAA
GAAGGAACCGGATTGGAAATCGGAGCAGCAGGATGATAATCAACTAAAGCAATGGATAGTGCCACTTA
TCCTGGGTCTGCTCGCAACCATCCTCTATCGGTTCTACTTCACCCAGCATCATCACCATCACCACTAA
AAGTT

H **Met** A E V K T Y S L A E V K S H N T N K S T W I V I H N D I F D V T E F L N E H P G G E E
V L L E Q A G K E A T E A F E D V G H S S D A R E **Met** **Met** K K F K V G E L I E S G R K Q V
P V K K E P D W K S E Q Q D D N Q L K Q W I V P L I L G L L A T I L Y R F Y F T Q **H H H H**
H H **Stop** K

Figure A5.2: Sequences of cytb_5(His)$_6$. Restriction enzymes are underlined, *Nde*I (before start codon) and *Hind*III (After stop codon). Six His –tag is indicated in blue.

A6: Nucleotide and Amino acid sequences of CPR

CCATGGCATCATCACCATCACCACAAGGGCAAGAAAAAAGAAAACCAAGCTAGTCAGTTCAAATCCTA
CTCGATCCAACCGACCACGGTAAACACGATGACAATGGTGGAGAATTCGTTCATCAAGAAGCTGCAAT
CCTCTGGTCGTCGTTTGGTGGTGTTCTACGGTTCTCAAACAGGCACGGCAGAGGAATTTGCCGGTCGT
CTGGCAAAGGAAGGAATCCGCTACCAGATGAAGGGCATGGTAGCCGATCCGGAGGAGTGCAATATGGA
AGAACTACTGATGTTGAAAGACATCGACAAATCGTTGGCTGTGTTTTGCTTAGCAACGTACGGTGAGG
GCGATCCGACGGACAACTGTATGGAGTTTTACGACTGGATTCAAAATAACGATCTGGATATGACCGGT
TTAAATTATGCGGTGTTTGGCCTGGGCAATAAAACGTACGAGCATTACAACAAGGTCGGCATCTATGT
TGACAAGCGGTTAGAGGAACTCGGAGCAAACCGAGTGTTTGAGCTAGGATTAGGCGACGATGATGCGA
ACATTGAGGATTACTTCATTACGTGGAAGGAAAAGTTTTGGCCAACGGTGTGCGATTTCTTCGGCATT
GAAAGTACGGGCGAGGATGTGTTGATGCGACAGTATCGTCTGTTGGAGCAACCGGATGTCGGTGCGGA
CCGTATATACACCGGTGAGGTGGCCCGGCTACACTCACTGCAGACGCAGCGCCCACCGTTCGATGCCA
AGAACCCGTTCCTAGCCCCCATCAAGGTAAACCGGGAGCTGCACAAGGCCGGGGACCGTTCCTGCATG
CACGTCGAGTTCGATATCGAGGGCTCGAAGATGCGGTACGAGGCCGGTGATCATTTGGCCATGTACCC
GGTGAACGATCGGGATCTGGTCGAGCGGCTCGGCAAGCTGTGCAACGCGGATCTGGAAACGATATTCT
CGCTGATCAACACCGACACGGACAGCAGCAAAAAGCATCCATTCCCCTGCCCGACCACCTACCGGACG
GCTTTGACGCACTACCTCGAGATAACGGCGCTACCCCGGACGCACATCCTGAAGGAGCTGGCCGAGTA
CTGCACGGAGGAGAAGGACAAAGAGTTCCTGCGGTTCATCTCGTCTACCGCGCCGGAAGGAAAGGCAA
AGTACCAGGAGTGGATACAGGACAGCAGCCGCAACGTGGTGCACGTGCTGGAGGATATCCCGTCCTGC
CATCCACCGATCGATCACGTGTGCGAACTGTTACCGCGTCTGCAGCCGCGCTACTATTCCATCTCCTC
CTCGTCCAAGCTCCACCCGACGACGGTACACGTGACGGCGGTGCTGGTGAAGTACGAGACGAAAACGG
GCCGATTGAACAAGGGTGTCGCTACGACATTCCTTGCCGAAAAGCACCCGAACGATGGGGAACCGTTG
CCGCGCGTACCTATCTTCATTCGCAAGAGCCAGTTCCGTTTGCCACCGAAACCGGAAACGCCCGTAAT
CATGGTCGGTCCTGGTACCGGGCTGGCACCGTTCCGTGGCTTCATACAGGAGCGTGACTTCTCCAAGC
AGGAGGGCAAGGAAATAGGGCAGACGACGCTATACTTTGGCTGCCGAAAGCGTACAGAGGACTACATA
TACGAAGACGAACTGGAGGATTATTCCAAACGTGGCATCGTTAACCTGCGCGTTGCCTTTTCGCGTGA
CCAAGAGAAGAAGGTGTACGTGACGCATCTGCTCGAGCAAGATAGCGACCTGATATGGAACGTTATCG
GCGAGAATAAGGGACACTTTTACATCTGCGGTGATGCGAAAAATATGGCCACCGATGTGCGAAACATT
CTGCTTAAAGTCATACGCTCAAAAGGTGGTTTAAGCGAAACGGAAGCACAGCAGTACATAAAAAAGAT
GGAAGCCCAAAAACGATACTCGGCGGACGTGTGGAGCTAAAGATCT

P W **H H H H H H** K G K K K E N Q A S Q F K S Y S I Q P T T V N T **Met** T **Met** V E N S F I K
K L Q S S G R R L V V F Y G S Q T G T A E E F A G R L A K E G I R Y Q **Met** K G **Met** V A D P
E E C N **Met** E E L L **Met** L K D I D K S L A V F C L A T Y G E G D P T D N C **Met** E F Y D W I
Q N N D L D **Met** T G L N Y A V F G L G N K T Y E H Y N K V G I Y V D K R L E E L G A N R
V F E L G L G D D D A N I E D Y F I T W K E K F W P T V C D F F G I E S T G E D V L **Met** R Q
Y R L L E Q P D V G A D R I Y T G E V A R L H S L Q T Q R P P F D A K N P F L A P I K V N R
E L H K A G D R S C **Met** H V E F D I E G S K **Met** R Y E A G D H L A **Met** Y P V N D R D L V
E R L G K L C N A D L E T I F S L I N T D T D S S K K H P F P C P T T Y R T A L T H Y L E I T
A L P R T H I L K E L A E Y C T E E K D K E F L R F I S S T A P E G K A K Y Q E W I Q D S S
R N V V H V L E D I P S C H P P I D H V C E L L P R L Q P R Y Y S I S S S S S K L H P T T V H V
T A V L V K Y E T K T G R L N K G V A T T F L A E K H P N D G E P L P R V P I F I R K S Q F
R L P P K P E T P V I **Met** V G P G T G L A P F R G F I Q E R D F S K Q E G K E I G Q T T L Y F
G C R K R T E D Y I Y E D E L E D Y S K R G I V N L R V A F S R D Q E K K V Y V T H L L E
Q D S D L I W N V I G E N K G H F Y I C G D A K N **Met** A T D V R N I L L K V I R S K G G L S
E T E A Q Q Y I K K **Met** E A Q K R Y S A D V W S **Stop** R S

Figure A6.1: Sequences of (His)$_6$CPR. Restriction enzymes are underlined, *Nco*I (before start codon) and *Bgl*III (After stop codon). Six His –tag is indicated in blue.

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